NEUROINFLAMMATION AND COMORBID DEPRESSION: A ROLE FOR INTERLEUKIN-1 BETA CONVERTING ENZYME AND INDOLEAMINE 2,3-DIOXYGENASE

BY

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DISSERTATION

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Abstract

Inflammation is a common component of numerous diseases and considerable evidence has linked components of inflammation with neuropsychiatric disorders. Major depression shares etiologies with inflammatory disease and some postulate that depression is an inflammatory disease. Inflammatory factors such as cytokines have been extensively correlated with depressive disorders in both clinical and experimental models. Whether depression leads to inflammation or inflammation increases the risk of depression remains an open ended debate where substantial evidence supports both sides of this argument. Important when considering the role that inflammation plays in depression, regardless of whether it is causative or resulting from products of inflammation, is that depression and inflammation have been linked. Depression is comorbid with numerous inflammatory diseases including diseases of the central nervous system. Individuals stricken with neurodegenerative diseases such as Alzheimer's, Parkinson's or human immunodeficiency virus (HIV) disease display a higher prevalence of comorbid depression during their life span. Common in these neurodegenerative disorders is inflammation within the central nervous system or neuroinflammation.

Neuroinflammation is recognized as an activation of microglia and astrocytes in nervous system tissues that leads to production of inflammatory mediators. Interleukin-1 beta (IL-1β) is a proinflammatory cytokine that elicits physiological and behavioral disturbances associated with both neuroinflammatory degenerative diseases and mood disorders. IL-1β requires enzymatic maturation by interleukin-1 beta converting enzyme (ICE) before being released as mature active cytokine. ICE activity is induced predominately during inflammatory events and deletion of ICE has previously been shown to be protective following lipopolysaccharides (LPS) administration. A series of studies were designed to test the hypothesis that ICE is necessary for development of depression-like behaviors in response to neuroinflammation induced by LPS. These studies

established that ICE in brain is necessary for depression-like behavior in response to immune stimulation induced by central administration of lipopolysaccharide (LPS). Moreover, these studies established that deletion of ICE prevented the sustained expression of brain cytokine mRNA that corresponded to protection against LPS-induced depression like behavior.

The tryptophan degrading enzyme indoleamine 2,3-dioxygenase 1 (IDO1) has also been implicated in neurodegenerative disease and depression. IDO1 activity increases kynurenine concentrations in circulation and brain tissue following LPS administration. Further, IDO1 has been shown to be necessary for the development of systemic inflammation-induced depression-like behaviors of mice. Increased brain kynurenine however, has not been linked to increased brain-specific IDO1 activity and depression-like behavior. A series of studies were conducted to investigate depression-like behavior and subsequent IDO1 activity of mice following a modest dose of LPS administered directly into brain. LPS increased kynurenine concentration specifically within brain that culminated in depression-like behavior. Genetic deletion of IDO1 or administering a pharmacological inhibitor, 1-methyl tryptophan, into the brain protected mice from LPS-induced depression-like behavior. These effects on behavior indicate that activation of brain IDO1 is necessary to induce depression-like behavior.

HIV disease is characterized by the infiltration of infected cells into the brain that results in many of the neurological disturbances observed during disease progression, known as neuroAIDS. Neuroinflammation represents a significant component to many HIV-associated neurological disorders. Further, increased prevalence of comorbid depression is observed with HIV infection. HIV proteins have drawn significant interest for their role in the inflammatory cascades that lead to neurodegenerative components of HIV disease. The HIV transactivator of transcription (Tat) protein is known to initiate an inflammatory response. A series of studies was

designed to determine whether acute brain administration of Tat induced neuroinflammation, brain cytokine and IDO1 expression that subsequently induced depression-like behavior. A single injection of Tat induced depression-like behavior that was accompanied by increased brain expression of proinflammatory cytokines and IDO1. Tat also induced mRNA expression of genes associated with activation of microglia and astrocytes. Taken together these findings indicate that Tat in the brain induces neuroinflammation and also induces depression-like behavior demonstrating a potential causative role for Tat in HIV comorbid depression.

These studies sought to characterize a role for both ICE and IDO1 in models of neuroinflammation comorbid depression induced by both LPS and HIV Tat protein. It was hypothesized that both of these molecules contribute to the development of neuroinflammation induced depression-like behavior. These findings confirm the hypothesis and establish that ICE and IDO1 represent potential therapeutic targets for alleviating comorbid depression associated with neuroinflammatory disease.

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Table of Contents

Chapter

1. Introduction and Justification 1
- 1.1 Literature Cited 3

2. Literature Review 5
- 2.1 Inflammation, sterile inflammation and neuroinflammation 5
- 2.2 Cytokines, sickness and depression 9
- 2.3 Interleukin-1 beta converting enzyme 13
- 2.4 Indoleamine 2,3-dioxygenase 18
- 2.5 Human immunodeficiency virus transactivator of transcription protein 20
- 2.6 Literature cited 23

3. Interleukin-1 beta converting enzyme is necessary for development of depression-like behavior following intracerebroventricular administration of lipopolysaccharides to mice 33
- 3.1 Abstract 33
- 3.2 Introduction 34
- 3.3 Materials and Methods 36
- 3.4 Results 40
- 3.5 Discussion 45
- 3.6 Figures 53
- 3.7 Tables 57
- 3.8 Literature Cited 58

4. Intracerebroventricular administration of lipopolysaccharide induces indoleamine 2,3 dioxygenase-dependent depression-like behaviors 62
- 4.1 Abstract 62
- 4.2 Introduction 62
- 4.3 Materials and Methods 64
- 4.4 Results 68
- 4.5 Discussion 70
- 4.6 Figures 75
- 4.7 Table 78
- 4.8 Literature Cited 79

5. Intracerebroventricular administration of HIV-1 Tat induces brain cytokine and indoleamine 2,3-dioxygenase expression: a possible mechanism for AIDS comorbid depression 82
- 5.1 Abstract 82
- 5.2 Introduction 83
- 5.3 Materials and Methods 85
- 5.4 Results 90

5.5 Discussion 92
5.6 Figures 97
5.7 Literature Cited 100

6. Summary and Future Considerations 104
6.1 Literature Cited 107

Chapter 1

Introduction and Justification

Advances in medicine, improved nutrition and changes in hygiene have all contributed to improved overall health, thus the average life span of individuals continues to increase (Wang et al. 2012). This increase in life expectancy among other factors may be contributing to the growing number of individuals that are being diagnosed with neurological and mood disorders that decrease the quality of life (Cowan and Kandel 2001). Of particular interest, an increasing number of individuals may be affected by Alzheimer's and Parkinson's disease, multiple sclerosis, human immunodeficiency virus (HIV) disease and major depression in their lifespan (Cowan and Kandel 2001). Neurodegenerative disease is accompanied by chronic inflammation within the brain in response to neuronal cell loss; a process termed neuroinflammation (Frank-Cannon et al. 2009, Wyss-Coray and Mucke 2002). Importantly, individuals impacted by chronic neuroinflammatory diseases have an increased likelihood of developing depression during their life span with the disease compared to non-affected individuals as evidenced by higher prevalence with neurodegenerative diseases such as multiple sclerosis (Feinstein 2011), Huntington's disease (Rosenblatt 2007), Parkinson's disease (Aarsland et al. 2012), HIV disease (Asch et al. 2003), and Alzheimer's disease (Aznar and Knudsen 2011).

Depression has been linked to numerous chronic inflammatory conditions with theories put forth that depression may either manifest or exacerbate disease or alternatively is comorbid with these chronic inflammatory conditions (Raison et al. 2006). Regardless of the precise role of depression, it is now increasingly accepted that neuroinflammatory stimuli such as chronic stress and neurodegenerative disease induce inflammatory processes that stimulate release of immune mediators that influence behavior and mood (Miller et al. 2009). Principal in these processes is

the release of pro-inflammatory cytokines. Release of these cytokines, particularly within the brain, mediates many physiological and neurovegetative symptoms that are common components of depression (Dantzer 2009).

Both depression and inflammation share common etiologies represented by the presence of cytokines and other immune mediators. Cytokines are produced by immune cells to aid in the isolation and elimination of threats posed by pathogenic microbes. However, it is important to consider that these molecules can often be detrimental to the host cells as well, particularly during chronic inflammatory events or when the threat is not eliminated by their production. The proinflammatory cytokine, interleukin-1 beta (IL-1β) has been extensively studied for its role in mediating neuroinflammatory events common to neurodegenerative disease, but also has been implicated in depression (Howren et al. 2009, Maes et al. 2012, Rothwell and Luheshi 2000). Suppression of the IL-1 system therefore has been and still remains an important target for therapies directed towards attenuating neuroinflammation and depression (Maes et al. 2012, Rothwell and Luheshi 2000).

Cytokines can also induce the production of other inflammatory mediators that are known to play a role in depression. In the last decade, increasing interest has focused on the tryptophan degrading enzyme indoleamine 2,3-dioxygenase (IDO1). IDO1 is the first and rate limiting enzyme in a metabolic cascade that leads to a transient increase in levels of kynurenine both in circulation and within tissues. Elevated IDO1 expression and increased concentrations of its product, kynurenine, have been correlated with inflammation associated comorbid depression (Myint et al. 2007). Importantly, many individuals impacted by chronic neuroinflammatory disease states such as Alzheimer's disease, Parkinson's disease and Huntington's disease along with mood disorders such as schizophrenia and major depressive disorder maintain elevated

levels of kynurenine pathway metabolites (Christmas et al. 2011, Myint 2012, Schwarcz et al. 2012). However, the precise role that brain IDO1 plays in neuroinflammation associated depression remains to be evaluated as studies to date have focused on investigating IDO1 and kynurenine metabolism using a whole body approach.

Gaining an understanding of which molecules play a role in mediating neuroinflammation-associated depression would provide significant benefit to those individuals who are affected. However, direct targeting of immune system molecules as a means to limiting neuropsychiatric disturbances may not be the best approach, as many of these molecules are important for normal physiologic function. Therefore, identifying and investigating the role of biological substrates associated with neuroinflammation-induced depression could pose insight into potential therapeutic targets that would allow for an improvement in quality of life in those affected.

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Chapter 2

Literature Review

2.1 Inflammation, sterile inflammation and neuroinflammation

Inflammation is a major component of many chronic disease states afflicting people throughout the world. Inflammation is classically characterized by the symptoms of calor (heat), rubor (redness), dolor (pain), turgor (swelling) and functio laesa (loss of Function). The inflammatory response is a necessary host defense mechanism employed by the body to control and clear infectious agents, thus attributing to its evolutionary importance. The classical symptoms of inflammation are outward and often visible signs of the actions of molecular components, i.e. cytokines, derived from immune cells following activation of the innate immune system. Under normal physiologic conditions, immune cells help to maintain homeostatic conditions by providing both pro-inflammatory and anti-inflammatory signals in response to various immune and non-immune stimuli.

Cells of the immune system which include monocytes, macrophages, neutrophils, dendritic cells, natural killer cells and B and T lymphocytes, work in concert to provide vital protection from numerous insults to normal physiological function. Molecular cues are necessary for activation of immune cells (Janeway and Medzhitov, 2002; Medzhitov and Janeway, 2002). These cues are classified into two broad categories, pathogen associated molecular patterns (PAMPs) and danger associated molecular patterns (DAMPs) (Bianchi, 2007). Molecular cues that come into contact with immune cells activate the first line of defense by ramping up immune activity to guard against bacterial, viral or parasitic insults that present these PAMPs that are recognized by these surveilling immune cells. Many PAMPs activate immune cells after binding to toll-like receptors (TLR) (Bianchi, 2007). Pattern recognition is achieved via these pattern

recognition receptors or TLR's using highly conserved microbe associated molecules such as the bacterial cell wall component lipopolysaccharide (LPS), flagellin, double stranded RNA, peptidoglycan and RNA variants that are encoded by viruses that leads to activation of the innate immune system (Ausubel, 2005). Immune cells also serve to initiate an immune response following recognition of endogenous molecules, which are not associated with pathogens that act as endogenous danger signals or DAMPs (Chen and Nunez, 2010; Rock et al., 2010). DAMPs, which are generally products of other host cells can initiate an immune response in the absence of infectious organisms (Chen and Nunez, 2010). Many DAMPs serve as a signal of tissue damage or are derived from cells that are displaying abnormal metabolic processes. Some notable DAMPs are proteins derived from cytosolic or nuclear origin including heat shock proteins, extracellular matrix proteins and high-mobility group box 1 (HMGB1) protein (Lotze et al., 2007; Rubartelli and Lotze, 2007; Kono and Rock, 2008). Small molecules that can present as DAMPs to immune cells include adenosine triphosphate (ATP) and other molecules usually associated with cellular metabolism, the nucleoside adenosine, and uric acid that is notable for its role in gout (la Sala et al., 2003; Shi et al., 2003; Kono and Rock, 2008). The presence of DNA outside of the nucleus of the cell may also serve as DAMPs (Kono and Rock, 2008; Pisetsky, 2012). Recognition of DAMPs like recognition of PAMPs is also achieved via pattern recognition receptors, notably TLRs, however nucleotide-binding oligomerization domain-like receptors (NLR) recognize intracellular pathogens (Janeway and Medzhitov, 2002) along with DAMPs, leading to innate immune activation (Kono and Rock, 2008; Mathews et al., 2008; Fukata et al., 2009). Activation of the innate immune system triggered by DAMPs is referred to as sterile inflammation since it is not triggered by a microbial source (Rock and Kono, 2008; Rock et al., 2010). Regardless of the source of DAMPs the immune system is activated but may

be ineffective since the source of molecular patterns is not of microbial origin and the inflammatory process may not solve the underlying issue or possibly may make it worse (Chen and Nunez, 2010; Rock et al., 2010). Regardless of whether immune activation is triggered from binding of PAMPs or DAMPs, the principal role mediated by secretion of immune effector molecules is killing of pathogens (Rock et al., 2010). Importantly, immune effectors can also have a destructive impact on host cells as well. Further, the damaging effects of prolonged inflammation that is associated with chronic elevations in inflammatory molecules can occur within the body without exhibiting the outwardly visible signs of inflammation such as in the case of obesity (Lee and Pratley, 2005), type 2 diabetes (Wellen and Hotamisligil, 2005), atherosclerosis (Libby et al., 2002), aging (Krabbe et al., 2004) and early stages of neurodegenerative diseases (McGeer and McGeer, 2004). Inflammation associated with these physiologic states, particularly brain inflammatory diseases poses a particularly difficult challenge since the ongoing inflammation plays a significant role in disease progression along with the associated comorbidities (Gao and Hong, 2008; Frank-Cannon et al., 2009).

The vast majority of research has focused on examining the mechanisms, interactions and prolonged effects of either acute and chronic immune challenges posed by microorganisms or molecules associated with these pathogens. However, numerous chronic disease conditions carry what is termed a sterile inflammatory component (Rock et al., 2010). Sterile inflammatory responses are characterized as inflammation triggered by physical trauma, exposure to various toxins, ischemia, and antigenic proteins released in the body (Rock et al., 2010). Research investigating sterile inflammation is relatively new area of interest that examines the role of immune system components in chronic disease states that are not directly linked to the presence of pathogens (Chen and Nunez, 2010; Rock et al., 2010). Unlike the outwardly visible

components of classical inflammation, sterile inflammation is mediated by molecular products of immune cells such as cytokines and other molecules that can have profound influence over the long term well-being and behavior of an organism (Kono and Rock, 2008; Anisman and Hayley, 2012).

Most neurodegenerative disease states, such as those observed in the brains of individuals suffering from Parkinson's and Alzheimer's disease, exemplify the processes associated with sterile inflammation in that the immune system and its components are intimately involved in the degenerative processes without direct induction from a microbial origin (Wyss-Coray and Mucke, 2002; Frank-Cannon et al., 2009). The brain is generally considered to be an immune privileged organ since it does not exhibit the classical signs of inflammation and is protected by a blood-brain barrier that separates the brain from molecules of peripheral circulation (Bechmann et al., 2007; Galea et al., 2007). Within these central nervous system (CNS) tissues, inflammation, termed neuroinflammation, formerly known as reactive gliosis, is characterized by increased glial activation and production of pro-inflammatory cytokines in response to tissue damage and or invasion by a pathogen (Streit et al., 2004). Additionally, neuroinflammation can cause increased blood-brain-barrier permeability that opens the brain to leukocyte invasion (de Vries et al., 1997; Petty and Lo, 2002). Neuroinflammation is a significant component of many neurodegenerative diseases and has been researched for its etiology involving the tissue destructive nature of these diseases (Wyss-Coray and Mucke, 2002; Frank-Cannon et al., 2009). Activation of glia and more specifically microglia, has been studied since the early 1900's as a component of neuroinflammation based on their phagocytic and antigen presenting properties observed during numerous neurodegenerative disease states (reviewed in (McGeer and McGeer, 2011)).

2.2 Cytokines, sickness and depression

The primary way in which immune cells interact with other cells of the body following immune stimulation is via the production of cytokines. Cytokines are generally classified as either being pro-inflammatory or anti-inflammatory in nature. Cytokines that are generally considered pro-inflammatory include but are not limited to interleukin (IL)-1β, IL-2, TNF-α, IL-6, IL-18 and interferon gamma (IFN-γ). Anti-inflammatory cytokines include IL-4, IL-10, IL-1 receptor antagonist (IL-1RA) and TGF-β along with receptors for IL-1β, TNF-α and IL-18 that act as a decoy receptors. Cytokines have multiple actions within the body that serve to intensify or dampen the immune response.

A major influence of cytokine actions on cells of the body is to stimulate the production of other inflammation associated molecules including eicosanoids, such as prostaglandins and leukotrienes, and glucocorticoids such as cortisol/corticosterone that have significant physiological impacts throughout the body including in brain. Further, both cytokines and these other inflammation associated molecules have significant influence in many chronic disease states including neuroinflammatory diseases, metabolic syndrome/obesity, cancer and aging. Increasing research importance is being placed on investigating how these inflammation-associated molecules influence the function of the brain. More specifically, increasing research focus is targeted at examining the output of the brain, which is principally responsible for our behavior and how we feel, i.e. our mood. In 1990, the summary of research at this time, led to the formulation of the macrophage theory of depression that focused on how chronic secretion of monokines (cytokines) due to numerous insults to normal physiological processes was driving the ever increasing prevalence of depression (Smith, 1991a). Ronald Smith noted in his theory the similarities between sickness and depression and extended his theory to conclude that IL-1

secreted from macrophages was responsible for depression. This theory took several factors into consideration including increased comorbid depression, higher prevalence in females due to estrogen activation of macrophages and also the increased dietary intake of omega-6 fatty acids. Smith's theories included theories of how immune system molecules influence numerous neurological conditions including schizophrenia, headache and other conditions including AIDS (Smith, 1991b, 1992b, a). Although this theory has been largely refined by other investigators to incorporate the additional inputs of other mediators, it provided an interesting launching point for expansion of research into how inflammatory mediators influence neuropsychiatric disease (reviewed in (Capuron and Dantzer, 2003; Irwin and Miller, 2007; Miller et al., 2009; Loftis et al., 2010)). Importantly, clinical data along with animal research studies conducted over the last decade has established that the prevalence of comorbid depression increases with chronic inflammation (Dantzer et al., 2008; Capuron and Miller, 2011).

Many of the visible signs of inflammation results from release of pro-inflammatory cytokines, however it is important to consider that ongoing inflammation can occur in the absence of these outward signs. This is often observed during periods of low-grade chronic inflammation such as in obesity, atherosclerosis and early neurological disease. Cytokine action is responsible for many behavioral responses that have been chronicled by years of intensive research that has exposed the negative association with chronic illness and neuropsychiatric disturbances such as major depression (Raison and Miller, 2011; Krishnadas and Cavanagh, 2012). The pro-inflammatory cytokines, IL-1β, TNF-α and IL-6 have all been implicated with increased fatigue, anorexia, and sleep in sick animals (Patarca, 2001; Kelley et al., 2003). Further, considerable evidence demonstrates how cytokines including IFN-α, IL-1β, TNF-α and IL-2 can induce behavioral symptoms of depression (Denicoff et al., 1987; Brebner et al., 2000;

Capuron et al., 2003b; Capuron et al., 2003a; Goshen et al., 2008; Kaster et al., 2012). An inflammatory state alters neuroendocrine systems (stress response, hypothalamic-pituitary-adrenal (HPA) axis activation) and provokes changes in dopamine and serotonin metabolism that have been and remain classical targets of antidepressant therapies (Connor and Leonard, 1998; Turnbull and Rivier, 1999). Furthermore, chronic stress can dramatically influence interactions between numerous physiologic systems in the body including the immune to brain communications (Dantzer and Kelley, 1989; Connor and Leonard, 1998). Additional evidence from research conducted using human subjects shows that a considerable link also exists between cytokines and depression (Maes et al., 1997; Levine et al., 1999; Himmerich et al., 2008; Howren et al., 2009). Two prominent examples have shown this to be the case. First, IL-6 is elevated in the serum of many depressed humans when compared to non-depressed controls (Maes et al., 1993; Maes et al., 1995; Frommberger et al., 1997; Maes et al., 1997). Second and most convincing is in patients given interferon alpha, an immunotherapy that is used for hepatitis treatment, the prevalence of major depression is nearly fifty percent based on DSM-IV criteria (Capuron et al., 2003b).

Many symptoms associated with human psychiatric disorders cannot be directly examined in animal models, therefore when investigating depression using animal models research is limited to examining behaviors that can be correlated to depression symptoms in humans (Nestler et al., 2002; Nestler and Hyman, 2010). Despite numerous limitations with the use of animal models to examine depression, animal behaviors correlated to depression symptoms at least offer predictive and in some cases face validity to this area of research (Nestler and Hyman, 2010). These behaviors, known as depression-like behaviors, are the research tool used to test for the presence of depression-associated symptoms based on the

consideration that emotionality or sadness cannot be directly assessed in animals. An indirect determination of the influence of cytokines for inducing depression-like behaviors is based on research using cellular components from bacteria that induces inflammation but without the complexities of an ongoing infection has shown similar results (Frenois et al., 2007; Godbout et al., 2008; O'Connor et al., 2009a; Park et al., 2011b). LPS, a cell wall component of gram negative bacteria, have been used to induce several behavioral disturbances. LPS induces cytokine expression that affect physiological functions leading to anorexia, disrupted sleep patterns, social withdrawal, decreased locomotor activity and other depression-like and anxiety behaviors (Kelley et al., 2003; Dantzer, 2009). Interestingly, many of these cytokine or LPS-induced behavioral changes can be attenuated with the administration of various cytokine antagonists, using mice with genetic deletion of numerous cytokines or their receptors or perhaps most compelling with the use of classic antidepressant drugs (Bluthe et al., 1992; Bluthe et al., 2000; Yirmiya et al., 2000; Yirmiya et al., 2001; Sparkman et al., 2006; Frenois et al., 2007). For example, many of the depression-like and anxiety behaviors induced via cytokine or LPS administration can be reversed with the administration of classic antidepressant drugs such as fluoxetine (selective serotonin reuptake inhibitor) (Frenois et al., 2007; Park et al., 2011a; Ohgi et al., 2013). Thus it is evident that cytokines and inflammation play a prominent in depression be it a causative relationship or whether depression increases inflammation and cytokines (Raison and Miller, 2011; Krishnadas and Cavanagh, 2012).

Numerous molecules that have been associated with depression in humans and depression-like behaviors in rodents are also components of inflammatory or neuroinflammatory events. Moreover, the prevalence rate of depression in patients suffering from neurodegenerative disorders is higher in these patients compared to the prevalence observed within the general

population. Individuals diagnosed with these diseases must cope with both the psychological stress impacts of battling diseases such as HIV (Asch et al., 2003), multiple sclerosis (Feinstein, 2011), Alzheimer's disease (Aznar and Knudsen, 2011), Parkinson's disease (Aarsland et al., 2012) or Huntington's disease (Rosenblatt, 2007) but also the influence of numerous biological substrates that may contribute to increased prevalence of depression in these patients. With regard to neuroinflammation, which is common to most neurodegenerative diseases, significant research has been directed towards the investigation of the IL-1 family (Rothwell and Luheshi, 2000; Basu et al., 2004; Allan et al., 2005). Of more recent interest, is the influence of the tryptophan catabolic pathway that leads to increased kynurenine and its downstream metabolites (Schwarcz et al., 2012). To this point, limited research has been devoted to investigating the role of the two endogenous enzymes indoleamine 2,3-dioxygenase 1 (IDO1) and interleukin-1 beta converting enzyme (ICE). ICE converts immature pro-IL-1β to the active mature IL-1β whereas IDO1 is the first and rate limiting enzyme for converting tryptophan to kynurenine. Importantly, both of these enzymes are present in brain and are induced in response to neuroinflammatory stimuli.

2.3 Interleukin-1 beta converting enzyme

Interleukin-1 beta converting enzyme (ICE) is an intracellular protein that has cysteine protease activity that cleaves its substrates following aspartic acid residues. ICE is synthesized as the inactive protein precursor or zymogen pro-ICE, which can be self-activated following stimulation by myriad of signals from microbial or endogenous origin. Activation results in proteolytic cleavage of pro-ICE into 10 and 20 kD subunits (Fantuzzi and Dinarello, 1999). These subunits form heterodimers that make up the mature enzymatically active ICE protein. ICE activity is essential for cleaving pro-IL-1β and pro-IL-18 into mature proteins (Fantuzzi and

Dinarello, 1999). ICE also plays a role in cleaving other pro-caspase proteins into mature proteins that play a role in cell death/survival.

Both cytokines that are processed by ICE activity are important for immune function. Mature IL-18 can synergize with IL-12 to activate T lymphocytes. IL-18, which is also labeled interferon-gamma inducing factor, stimulates both T lymphocyte and natural killer cells to produce IFN-γ and increases cytolytic activity of natural killer cells (Alboni et al., 2010). Upon cleavage by ICE, IL-1β plays an important role in stimulating immune cells by triggering immune reactions that lead to cellular activation/recruitment and increased cytokine production. IL-1β is a member of what is termed the IL-1 superfamily of cytokines (Sims and Smith, 2010). It is secreted as a 30 kD precursor pro-IL-1β that is processed to a 17 kD mature secreted protein. The behavioral and molecular changes induced by IL-1β are driven principally by the downstream actions following binding of IL-1β to the type 1 IL-1 receptor (IL-R1). Signaling of IL-1R1 is conducted through a series of protein kinase reactions to activate nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and other stress kinases. Initiation of IL-1R1 binding and signaling requires its coupling with a secondary IL-1 receptor accessory protein (IL-1RAP). A second receptor, type-2 IL-1 receptor (IL-1R2), can also bind to IL-1R1 but does not induce signaling thus inhibiting actions of IL-1β by blocking binding to IL-1R1. In addition to inhibition mediated through IL-1R2, an antagonist protein conveniently labeled IL-1 receptor antagonist (IL-1RA) plays a prominent role in inhibiting actions of IL-1β. Many of the behavioral deficits induced with IL-1β can be opposed by co-administration of IL-1RA. Importantly all of these proteins are expressed in brain indicating that IL-1β can both signal and be inhibited within the CNS without influence from the periphery.

ICE activation is dependent upon the assembly of a complex of proteins known as inflammasomes. These protein complexes consist of ICE, apoptosis-associated speck-like protein containing a c-terminal caspase recruitment domain (ASC) that acts as an adaptor protein that contains a c-terminal caspase recruitment domain (CARD) and an n-terminal pyrin adaptor domain (PYD) (Schroder and Tschopp, 2010). Inflammasomes also contain Nod-like receptor proteins (NLRP) that give inflammasomes specificity to various stimuli such as PAMPs and DAMPs as well as provide a nomenclature for differentiating the inflammasome protein complexes (Schroder and Tschopp, 2010). Inflammasomes appear to have limited specificity to various stimuli with multiple inflammasomes responding to the same stimulus and in some cases multiple stimuli are needed to gain full inflammasome activation of ICE (Franchi et al., 2009). The NLRP3 inflammasome appears to be most influential for activating ICE following LPS treatment (Franchi et al., 2009). Activation of NLRP3 inflammasomes following LPS may be mediated by a combination of TIR-domain-containing adapter-inducing interferon-β (TRIF) activation following LPS binding of toll-like receptor 4 (TLR4) and presence of extracellular molecules such as ATP activate ATP-gated purinergic receptor P2X, ligand-gated ion channel, 7 (P2X7R) (Franchi et al., 2009). The precise mechanism for NLRP3 inflammasome activation has not been completely elucidated as many stimuli can interact with the NLRP3 protein but ATP appears to be the most potent inducer.

The current hypothesis for activation of inflammasomes and IL-1β production following stimulation with LPS which is principally located within macrophage, monocytes and microglia (Ayala et al., 1994; Yao and Johnson, 1997) within the brain is not well defined as LPS alone does not induce large secretion of IL-1β (Franchi et al., 2009)}. However it is well accepted that following LPS stimulation TLR4 binding leads to increased transcription of pro-inflammatory

cytokine genes including pro-IL-1β. Importantly, LPS induces the gene expression of inflammasome proteins, including ICE and NLRP3, potentially leading to an increased numbers of assembled inflammasomes that ultimately leads to increased processing of IL-1β (Schroder and Tschopp, 2010). One potential mechanism detailing how LPS induces inflammasome/ICE activation follows that priming of myeloid lineage cells with PAMPs such as LPS, increases NFκB that is necessary to promotes transcription and synthesis of inflammasome components (Lamkanfi and Dixit, 2009; Netea et al., 2010). Subsequently, the release of proinflammatory cytokines then stimulates the release of ATP that leads to opening of pannexin channels following binding with P2x purinoceptor 7 and inflammasome assembly that results in the activation of ICE thus increasing active IL-1β and IL-18 for secretion (Kahlenberg et al., 2005; Franchi et al., 2009).

IL-1β is considered to be a pro-inflammatory cytokine and when released from activated immune cells has numerous influences on the central nervous system (Rothwell and Luheshi, 2000; Basu et al., 2004). IL-1β administered systemically or directly into brain can induce transient sickness associated behaviors such as fever, anorexia, and decreased social investigation (Kent et al., 1992; Burgess et al., 1998; Neveu et al., 1998; Yao et al., 1999). Sickness associated physiological and behavioral changes can be attributed to IL-1β influence on molecular stress systems within the brain and peripheral tissues (Besedovsky et al., 1991). Elevations in IL-1β from exogenously administered IL-1β, tissue damage or as a consequence of immune stimulation can activate the HPA axis (Besedovsky and del Rey, 2000). This IL-1β induced activation of HPA axis leads to increased plasma glucocorticoids and catecholamines (Berkenbosch et al., 1989). IL-1β can also induce cyclooxygenase 2 leading to increased production of prostaglandin E2 (PGE2) (Basu et al., 2002). Prostaglandins are eicosanoids that

are derived from oxidative metabolism of arachidonic acid. Elevated levels of eicosanoids have been implicated in many chronic inflammatory diseases including neurodegenerative diseases (Ricciotti and FitzGerald, 2011; Lima et al., 2012). Ultimately these compounds along with the increase in cytokines and other inflammation associated factors such as prostaglandins, impact areas of the central nervous system that trigger physiological responses often visible as outward signs of stress and/or sickness (reviewed in (Turnbull and Rivier, 1999)).

As stated earlier, cytokine expression within the CNS has many impacts on behavior (Dantzer, 2009). Given that IL-1β is upregulated in brain tissue in patients suffering from neurodegenerative diseases including Parkinson's disease, multiple sclerosis and Alzheimer's disease, it is plausible that IL-1β plays an intricate role in comorbid depression associated with neuroinflammatory conditions (Maes et al., 2012). Increased IL-1β observed in neurodegenerative diseases are likely driven by inflammasome recognition of damage associated molecular patterns (DAMPs) such as ATP and amyloid beta following apoptotic events associated with these diseases (Allan et al., 2005). Inflammasome activation leads to activation of ICE that in turn leads to elevations in brain IL-1β. IL-1β in the brain is not just associated with negative impacts on brain tissue. The presence of IL-1β in brain is now regarded as a necessary component of normal brain function as its role in learning has been demonstrated (Yirmiya et al., 2002). Administration of IL-1RA impairs spatial learning whereas additional IL-1β had no effect on learning. However, exogenous IL-1β intraventricular injections improved memory in a passive avoidance task (Yirmiya et al., 2002). Since direct targeting of IL-1 signaling may not be a viable option for treating depression comorbid with neuroinflammation, ICE may pose as a viable alternative target since its activity is predominately linked to increased inflammatory stimuli via inflammasome activation (Denes et al., 2012).

2.4 Indoleamine 2, 3-dioxygenase

Common components of neurodegenerative disease include inflammatory processes that stimulate release of immune mediators that influence behavior and mood. Release of cytokines, particularly within the brain, mediates the physiological and neurovegetative symptoms that share commonality in both sickness and depression. During periods of acute and also chronic immune stimulation, secreted cytokines stimulate the production of molecules that have been associated with depression (Dantzer et al., 2008). Of particular interest is the tryptophan degrading enzyme IDO1, as elevated IDO1 activity and its product kynurenine have been correlated with inflammation induced depression (Dantzer et al., 2011). Both the activity and genetic expression of IDO1 is stimulated by pro-inflammatory cytokines including IFN-γ, TNF-α, and IL-1β (Bianchi et al., 1988; Babcock and Carlin, 2000; Fujigaki et al., 2001). Research has identified IFN-γ as the most potent inducer of IDO1 expression and activity (Ozaki et al., 1988; Taylor and Feng, 1991) while TNF-α and IL-1β have been shown to have a lower stimulatory effect on IDO1 induction comparatively (Babcock and Carlin, 2000).

IDO1 is the first and rate limiting enzyme in a metabolic cascade that leads to a transient increase in levels of kynurenine both in circulation and within tissues. IDO1 and the recently characterized IDO2 (Ball et al., 2007; Ball et al., 2009) along with the liver associated tryptophan 2, 3-dioxygenase are metabolic enzymes that degrades tryptophan to n-formyl-kynurenine that is then quickly converted to kynurenine. Subsequent metabolism of kynurenine via the kynurenine metabolic pathway in many cell types within the body typically leads to nicotinamide adenine dinucleotide (NAD) production as part of normal energy metabolism. However during times of heightened or prolonged inflammatory states the formation of kynurenine pathway intermediates including 3-hydroxy-kynurenine (3HK), quinolinic acid (QA),

and kynurenic acid (KA) that results in accumulating levels of these "neuroactive" metabolites that contributes to various disease states. Interestingly, kynurenine is not known to have direct effects on neuronal function but this remains an area of ongoing research (Stone et al., 2013). Thus QA, KA and 3HK have been targets of increasing research as potential mediators of inflammation induced depression (Schwarcz et al., 2012). Many chronic disease states have elevated levels of kynurenine pathway metabolites that have been implicated in neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease and Huntington's disease along with mood disorders such as schizophrenia and major depressive disorder (Zadori et al., 2009; Schwarcz et al., 2012).

Tryptophan metabolic products maintain increasing research interest for treatment of depression (Myint et al., 2012). The tryptophan metabolite serotonin has long been the target of depression research and selective serotonin reuptake inhibitors remain the most prescribed type of antidepressant (Dantzer et al., 2011). In addition to serotonin, most antidepressant therapies still continue to target the monoamine neurotransmitter systems, despite growing evidence that indicates that kynurenine metabolism may be a viable alternative target for some types of depression (Li et al., 2011). An important consideration validating the need for increased focus on IDO1/kynurenine pathway is that IDO1 enzymatic activity metabolizes both tryptophan and serotonin into kynurenine that then acts a substrate for downstream processing into the neuroactive metabolites (Stone and Darlington, 2002).

Previous work has demonstrated that IDO1/kynurenine pathway as an alternative hypothesis for driving the development of depressive episodes associated with acute inflammatory events as well as chronic inflammatory states (Andre et al., 2008; Godbout et al., 2008; Moreau et al., 2008; O'Connor et al., 2009a). It has been demonstrated that both inhibition

of IDO1 activity or genetic deletion of IDO1 abrogates inflammation associated depression-like behavior in a murine model of acute and chronic inflammation induced by LPS (O'Connor et al., 2009a) or Bacille Calmétte Guerin (BCG) (O'Connor et al., 2009b) respectively. Furthermore, increasing kynurenine levels in the periphery even in the absence of inflammation is sufficient to induce depression-like behaviors (O'Connor et al., 2009a; Salazar et al., 2012). However, it has yet to be established as to whether inflammation confined to brain and subsequent brain specific IDO1 induction following LPS administered directly into the brain necessary or sufficient to induce depression-like behaviors in mice. Recently, Dobos et. al. demonstrated that mice given intraventricular injections with LPS have increased depression-like behavior that is attenuated by chronic exposure to 1-methyl tryptophan that acts as a competitive inhibits IDO1 activity (Dobos et al., 2012). However, in this particular model the dose of LPS used was large enough that the authors suspected that LPS had effects on peripheral tissues and thus was not confined within the brain. It remains to be elucidated as to whether increased IDO1 activity in brain is capable of increasing brain kynurenine levels resulting from a neuroinflammatory challenge; in the absence of increased circulating kynurenine levels; whether this increase was capable of inducing depression-like behavior. These are important considerations when evaluating whether IDO1 and kynurenine metabolism has significant impacts on the development of neurodegenerative disease in addition to the role that they may play in comorbid neuropsychiatric disturbances that often accompany these diseases.

2.5 Human immunodeficiency virus transactivator of transcription protein

The prevalence of human immunodeficiency virus (HIV) disease remains elevated despite a world-wide education effort targeted to reduce the continued spread of HIV. The United States Center for Disease Control (CDC.gov, 2010) estimates that there are more than 1

million infected persons in the United States with more than 200,000 being unaware of their infection status. Importantly, HIV infected individuals also have a higher prevalence of comorbid depression compared non-infected individuals (Asch et al., 2003). Although the prevalence rate of HIV comorbid depression varies based on various data obtained from numerous research studies, it is generally accepted based on meta-analysis that HIV comorbid depression occurs at twice the prevalence of a comparison group (Rabkin, 2008; Ownby et al., 2010). Depression in HIV infected individuals poses a significant problem for both the infected individuals but also the non-infected population. HIV infected depressed patients demonstrate a reduced adherence to drug compliance that allows viral particle counts to remain elevated compared to medicated patients (Yun et al., 2005). Further, depressed HIV patients often engage in illicit behaviors such as sharing drug needles or unprotected sex that may increase the risk of spreading HIV disease to uninfected persons (Stein et al., 2003; Diamond et al., 2005). One important factor to consider however is that even drug compliant patients have increased prevalence of comorbid depression when compared to the general population (Yun et al., 2005). Although significant research has been conducted to elaborate the role of immune system molecules for depression symptoms associated with HIV disease such as increased brain proinflammatory cytokines, HIV viral proteins have not been extensively investigated for their association with increased risk for depression in HIV disease.

The HIV protein Transactivator of Transcription (Tat) is of particular interest based on its unique role in HIV disease and its direct effects on brain immune cells. Tat is one of the first proteins produced in virally infected cells as it is necessary for increasing expression of viral proteins for viral replication (Romani et al., 2010). Interestingly, Tat remains to be ubiquitously expressed in all infected cells even during periods of low viral replication or latent phase of HIV

infection (Nath, 2002). HIV, a lentivirus, infects CNS tissue early on in HIV disease and remains latent in these tissues principally in macrophage and microglia following the primary phase of infection (Garden, 2002; Yadav and Collman, 2009). During viral latency and despite ongoing HIV targeted drug therapy, Tat continues to be produced in CNS tissues where it is known to increase expression of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1β (Nath et al., 1999; Sheng et al., 2000; Fu et al., 2011).

Tat has been studied principally for its contributions to HIV associated dementia (HAD) (Kim et al., 2003; King et al., 2006; Fitting et al., 2010). HAD is part of a complex of neurological disorders that are caused by HIV infection, collectively termed NeuroAIDS (Power et al., 2009). However, Tat has not been investigated for its role in HIV associated neuropsychiatric disturbances, specifically depression. Beyond its role for inducing neurodegenerative effects that are at least partially mediated by proinflammatory cytokines, it is possible that Tat may directly or indirectly induce HIV comorbid depression. An important consideration is that Tat may induce depression-like behavior via stimulation of brain immune cells and subsequent production of proinflammatory cytokines. Tat induced cytokines may promote depression directly or in combination with Tat or cytokine induced expression of other inflammatory mediators such as IDO1. Tat exposure has not been shown to induce depression in either animal models or clinical settings; however Tat does induce IDO1 expression in CNS tissues as well as in dendritic cell cultures (Samikkannu et al., 2010; Fu et al., 2011). Since cytokines and IDO1 have been demonstrated to be significant mediators of inflammation associated depression, it is plausible to consider that Tat expression during HIV infection along with Tat-induced cytokine and IDO1 expression is a potential mechanism to explain increased prevalence of HIV comorbid depression. A direct association with Tat induction of cytokines,

IDO1 and depression-like behaviors would further implicate these molecules as potential biological substrates driving depression during HIV disease.

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Chapter 3

Interleukin-1 beta converting enzyme is necessary for development of depression-like behavior following intracerebroventricular administration of lipopolysaccharides to mice

3.1 Abstract

Interleukin-1 beta converting enzyme (ICE, caspase 1) is a cysteine protease that processes immature pro-IL-1β into active mature IL-1β. IL-1β is a proinflammatory cytokine that mediates many of the physiological and behavioral responses to inflammation. Genetic deletion of ICE has previously been shown to prevent some negative physiologic responses to lipopolysaccharides (LPS)-induced inflammation. Here we used a preclinical murine model to test the hypothesis that ICE is necessary for development of depression-like behaviors following intracerebroventricular (ICV) treatment with LPS. Adult male ICE knockout (ICE KO) and congenic wild type C57BL/6J (WT) mice were administered LPS either ICV at 100 ng/mouse or intraperitoneally (IP) at 830 μg/kg body weight or an equal volume of saline as controls. Mice were monitored up to 48 h after treatment for both sickness and depression-like behaviors. LPS given ICV induced a loss of body weight in both WT and ICE KO mice. This sickness response was similar between WT and ICE KO mice. As expected, LPS administered ICV increased immobility in the forced swim test (FST) and decreased sucrose preference in WT mice but no change in either of these two depression-like behaviors was observed in ICE KO mice. Expression of TNF-α and CD11b in brain was lower in ICE-KO mice at 24 h following ICV administration of LPS compared to WT mice. In contrast, when LPS was given systemically, sickness response, depression-like behaviors and expression of these genes were similar between the two strains of mice. These findings indicate that ICE plays a specific role in depression-like

behavior induced by a central inflammatory stimuli even though it is not required when LPS is administered systemically.

3.2 Introduction

During the past two decades, substantial support has accumulated for the idea that the comorbid symptoms associated with clinical depression and neurodegenerative diseases share inflammation as a common an important component of their etiology (Smith, 1991; Raison and Miller, 2011; Beumer et al., 2012). Moreover, prevalence rates for neuropsychiatric disorders along with chronic inflammatory diseases, such as type II diabetes and heart disease within the general population, are rapidly increasing (Moussavi et al., 2007; Bodenheimer et al., 2009). Similarly, the incidence of neurodegenerative diseases such as Alzheimer's and Parkinson's is rising, predominately due to increasing life-span (Brookmeyer et al., 2007; Hindle, 2010). As a result of these challenges, therapies targeted to alleviate inflammation are now viewed as potential treatments for clinical depression (Catena-Dell'Osso et al., 2011).

Preclinical research using animals to model human depression has provided significant insight into which inflammatory mediators may be good therapeutic drug targets (Rothwell and Luheshi, 2000; Goshen et al., 2008; O'Connor et al., 2009b; O'Connor et al., 2009a). The overall goal is to diminish inflammation by blocking the production of inflammatory cytokines such as IL-1β (Miller et al., 2009; Maes et al., 2012). IL-1β plays a prominent role in neurodegenerative processes (Rothwell and Luheshi, 2000; Dinarello, 2011) and has recently been identified for its role in murine models of depression-like behavior (Goshen et al., 2008; Norman et al., 2010). In agreement with these preclinical models, evidence from human studies indicates higher cerebral spinal fluid IL-1β levels in patients suffering from acute depression (Levine et al., 1999).

Systemic administration of lipopolysaccharide (LPS) induces expression and activity of interleukin-1ß (IL-1β) and IL-1β-converting enzyme (ICE) in myeloid-derived cells that are resident to many organs, including brain microglia (Li et al., 1997; Yao and Johnson, 1997). ICE is the primary enzyme responsible for cleavage of pro-IL-1β and pro-IL18 to fully processed mature cytokines, eventually leading to increased secretion of active IL-β and IL-18. ICE activation is linked to assembly and activation of the inflammasome following recognition of numerous pathogen- or danger-associated molecular patterns and toll-like receptor binding (Franchi et al., 2009; Fleshner, 2012; Maslanik et al., 2012). ICE activity has been shown to influence food intake during inflammation (Burgess et al., 1998; Yao et al., 1999), presumably through its role in processing IL-1β. Evidence from experiments utilizing ICE KO mice demonstrated that these mice have impaired processing of pro-IL-1ß and reduced secretion of IL-1β following stimulation with LPS (Li et al., 1997). IL-1β plays a prominent role in brain during inflammation as first evidenced by experiments that demonstrated IL-1β-induced activation of hypothalamic-pituitary-adrenal axis and stress responses (Besedovsky and del Rey, 1988, 2000; Goshen and Yirmiya, 2009). Further, ongoing research continues to highlight the influence of IL-1β in many chronic inflammatory diseases and mood disorders. Thus targeting of ICE represents a possible alternative therapeutic route to target IL-1β.

We examined the role of ICE in depression-like behaviors utilizing murine models of systemic and central inflammation following an IP (systemic) or ICV (central) injection of LPS, respectively. Based on our previously published results (Burgess et al., 1998; Yao et al., 1999), we hypothesized that ICE KO mice would be protected from depression-like behavior induced by centrally administered LPS but not from depression-like behaviors induced by systemic LPS. Here we report that ICE KO mice were protected from central inflammation-induced depression-

like behavior as measured by two well-accepted behavioral tests (Nestler and Hyman, 2010). However, when challenged with systemic LPS, ICE KO mice displayed depression-like behaviors comparable to WT mice. This disparate behavioral response corresponds to decreased brain expression of proinflammatory cytokines and markers of glial activation in ICE KO mice following ICV but not IP LPS. Consequently, therapies designed to inhibit ICE activity may be a viable treatment of comorbid depression associated with inflammatory diseases of the central nervous system.

3.3 Materials and methods

Animals

C57BL/6J (WT) mice were purchased from Jackson Laboratories (Stock #000664, Bar Harbor, ME). ICE KO mice on a C57BL background were kindly provided by Dr. Richard Flavell (Yale University School of Medicine (Kuida et al., 1995)). These mice are genetically identical to mice now also available from Jackson Laboratories (Stock #016621). In these experiments, WT and ICE KO male mice were individually housed and provided ad libitum access to Teklad 8640 chow and water in a temperature- (23 °C) and humidity- (45%) controlled room and maintained on a 12:12 hour light:dark cycle (lights off at 10:00 am). Mice were acclimated to these conditions for a least 2 wk prior to initiation of any procedure. When mice reached at least ten weeks of age, those mice to be treated ICV were surgically implanted with a guide cannula (Plastics One, Roanoke, VA) directed toward the lateral ventricle as previously described (Lawson et al., 2011). The coordinates for implantation were determined utilizing The Mouse Brain in Stereotaxic Coordinates (Fanklin, 2001) and cannulas were placed at 1.5 mm lateral, 0.6 mm posterior, and 1.3 mm dorsal with respect to bregma. These coordinates placed the guide cannula 1 mm dorsal to the lateral ventricle. Mice were allowed to recover from

surgery for ten to fourteen days before being treated. Prior to any treatment mice were handled daily to habituate them to being restrained and manipulated. All procedures performed on the mice were in compliance with the National Institutes of Health guidelines and approved by the University of Illinois at Urbana Champaign Institutional Animal Care and Use Committee.

Treatments

Body weights were measured on the day of treatment prior to injections and 24 h later to evaluate sickness response. Mice were injected using a single internal injector cannula for mice (Plastics One, Roanoke, VA) which extended 1 mm beyond the tip of the guide cannula to reach the lateral ventricle. Mice were injected at the onset of the dark cycle using a 10 µl gas-tight syringe (SGE Incorporated, Austin, TX) to administer 1 µl of endotoxin free phosphate buffered saline (PBS) or 100 ng LPS (from Escherichia coli O127:B8, Sigma Aldrich, St Louis MO) in PBS. The injector cannula was left in place for approximately thirty seconds to allow for diffusion before dummy cannulas were placed back in guide cannulas.

Mice treated peripherally were weighed as described for central LPS studies. Mice were treated IP with either endotoxin-free injectable saline or LPS (830 µg/kg body weight) mixed with injectable saline.

Locomotor Activity

To evaluate the effects of saline or LPS on exploratory locomotor activity, mice were tested 24 h after treatment. Mice were placed in clear plexiglass cages identical to their home cage but devoid of bedding or nesting material. Clear plexiglass lids were placed on top of test cages to prevent escape while facilitating video recording of mice. Locomotor activity was assessed by virtual division of the cage into 4 equal quadrants and then tallying the number of

line crossings and rearings each mouse displayed during the five-minute test period. Videos were scored by trained personnel blinded to treatment.

Forced Swim Test

To determine whether LPS injected ICV or IP induced differential depression-like behaviors of ICE KO mice compared to WT mice, we utilized a modified version of the Porsolt Forced Swim Test (Porsolt et al., 2001). Mice were placed in a white plastic container (20 cm diameter x 24 cm tall) that was partially filled with 24 ± 0.5° C water. Test duration was five minutes and the mice were video recorded for analysis. Videos were scored by trained personnel blinded to treatment. Time of immobility was defined as the time when the mouse's effort was only that necessary to remain afloat. The forced swim test was administered 24 h after treatment.

Sucrose Preference Test

To quantify inflammation-induced anhedonia, which is a common symptom of major depression, we subjected mice to the two-bottle sucrose preference test. This test measures preference for sweetened solution over water. Approximately one week prior to treatment, mice were trained by simultaneous presentation with a bottle of water and a bottle of 1% (wt/vol) sucrose solution. Bottles were weighed prior to being placed on the lid of the mouse's home cage and left in place for 24 h periods. Mice were allowed ad libitum access to the bottles. After 24 h, the bottles were reweighed to determine the amount of sucrose solution and water that had been consumed. Preference was calculated as a percentage of sucrose solution consumed compared to the total fluid intake (sucrose/(sucrose + water) * 100). Mice were trained until a stable baseline preference was established and then treatments were administered. Following treatment, sucrose preference testing was conducted 24 to 48 h following treatment. This time frame corresponded

with amelioration of overt sickness response and presence of depression-like behavior, as assessed by the FST.

Tissue Collection

At either 4 or 24 h post-injection, mice were euthanized by CO2 asphyxiation. Brains were removed and longitudinally cut into hemispheric sections and immediately frozen in sample tubes placed on dry ice. The tissue was stored frozen at -80 °C until processing.

Tissue Processing and Quantitative Real Time RT-PCR (qRT-PCR) Analysis

Expression of cytokines and genes associated with immune activation was measured in brain to determine if ICE KO mice had differential pro- or anti-inflammatory responses to LPS compared to WT mice. One hemisphere of each brain was removed from storage and 3 ml of cold Trizol reagent (Invitrogen, Carlsbad, CA) were added to each sample. The tissue was then homogenized using an ultrasonic tissue disruptor (Sonics and Materials Inc., Newborn, CT). An E.Z.N.A kit was used to isolate total RNA (Omega Bio-tek, Norcross, GA). RNA purity (OD 260/280) and quantity was assessed using a Nanodrop Spectrophotometer (Nanodrop Products, Wilmington, DE) and submitted to reverse transcription using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). The cDNA samples were analyzed using qRT-PCR with the Prism 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). TaqMan gene expression assays (Applied Biosystems, Foster City, CA) or PrimeTime qPCR assays (Integrated DNA Technologies, Coralville, IA) were used for the detection of IL-1β/ pro-IL-1β (catalog no. Mm00434228_m1), IL-1RA (Mm00446186_m1), ICE (Mm.PT.49a.21858521), IL-18 (Mm00434225_m1), TNF-α (Mm00443260_g1), IL-6 (Mm00446190_m1), IL-10 (Mm00439614_m1), CD11b (Mm00434455_m1), MHC II (Mm00439226_m1), GFAP (Mm00546086_m1) and GAPDH (Mm99999915_g1). All assays

except for ICE and IL-1R1 were purchased from Applied Biosystems. Samples were analyzed in duplicate using 125 ng of cDNA template mixed with Taqman Universal Master Mix and target primers for each reaction according to the manufacturer's instructions. Relative quantitative measurement of target gene levels was performed using the □□Ct method, where Ct is the comparative threshold concentration. GAPDH was used as the endogenous housekeeping control gene to which all other genes were compared.

Statistical Analysis

Data are represented as the means ± SEM. All measures were analyzed using two-way analysis of variance (ANOVA). When the two-way interaction p value was less than 0.05, post-hoc analysis using Fishers protected least significant difference test was employed to test for differences among means.

3.4 Results

Sickness responses were similar in WT and ICE KO mice following central LPS injection.

LPS administered ICV decreased body weight of both WT and ICE KO mice (Table 3.1) over the 24 h period following treatment. Only the main effect of LPS was statistically significant (LPS main effect, $F_{1,24} = 45.50$, $p < 0.01$). Locomotor activity was tested 24 h following treatment (Table 3.1). LPS, given ICV, did not affect line crossings (LPS main effect, $F_{1,26} = 0.28$, $p > 0.05$) and rearings (LPS main effect, $F_{1,26} = 0.002$, $p > 0.05$) of either strain 24 h after treatment. However, ICE KO mice displayed reduced line crossings (strain main effect, $F_{1,26} = 27.50$, $p < 0.01$) and rearings (strain main effect, $F_{1,26} = 21.62$, $p < 0.01$) compared to WT mice. Despite the baseline difference in activity between the two strains, these responses indicate that deletion of ICE does not change the sickness response when LPS is administered centrally.

ICE KO mice were protected from central LPS-induced depression-like behavior.

To test the hypothesis that ICE KO mice would be protected from central LPS-induced depression-like behavior, we employed the FST and sucrose preference test following ICV treatment with LPS or saline. Importantly and in agreement with our hypothesis, LPS increased (strain x LPS interaction; $F_{3,24} = 4.35$, $p < 0.05$) immobility during the FST (Fig. 3.1A) of WT but not ICE KO mice. Similarly, ICE KO but not WT mice maintained (strain x LPS interaction; $F_{3,40} = 6.56$, $p < 0.05$) their preference for a 1% sucrose solution over water following LPS treatment (Fig. 3.1B). Taken together, these data confirm that ICE is necessary for expression of depression-like behavior following central administration of LPS.

Steady-state expression of brain inflammatory markers declined faster in ICE KO compared to WT mice.

To identify underlying substrates in ICE KO mice that are related to them being protected from ICV LPS-induced depression-like behavior, we utilized qRT-PCR to measure expression of cytokines and markers of active glia in brain collected 4 and 24 h after treatment. As expected, LPS induced ($p < 0.01$; data not shown) expression of ICE in WT mice at both time points although central LPS did not change IL-18 expression (data not shown).

To determine whether ICE deletion reduces neuroinflammation, we examined brain expression of pro-inflammatory cytokines (Fig. 3.2A). LPS increased (LPS main effect; $F_{1,22} = 91.63$, $p < 0.01$) IL-1β expression in both WT and ICE KO mice at 4 h indicating that LPS induces a similar initial inflammatory response in both strains. However, at 24 h, expression of IL-1β remained elevated (strain x LPS interaction; $F_{3,20} = 10.09$, $p < 0.01$) only in WT mice. LPS-treated ICE KO and WT mice had increased (LPS main effect; $F_{1,22} = 4.36$, $p < 0.01$) brain expression of TNF-α mRNA at 4 h compared to controls, but TNF-α expression returned (strain x LPS interaction; $F_{3,20} = 5.72$, $p < 0.05$) to control levels in ICE KO mice by 24 h after treatment

while TNF-α expression remained elevated in WT mice. At 4 h after treatment, LPS induced IL-6 expression (strain x LPS interaction; $F_{3,20} = 10.19$, $p < 0.05$) but the LPS response was smaller in ICE KO mice compared to LPS-treated WT mice. At 24 h, brain IL-6 expression was increased by LPS (LPS main effect; $F_{1,22} = 5.52$, $p < 0.05$). However, ICE KO mice tended (strain x LPS interaction; $F_{3,20} = 3.84$, $p = 0.06$) to have reduced brain IL-6 expression 24 h after ICV injection of LPS.

We examined expression of IL-10 and IL-1RA within the brain to determine if ICE deletion leads to increased expression of anti-inflammatory genes (Fig. 3.2B). IL-10 mRNA increased similarly in both strains at 4 (LPS main effect; $F_{1,22} = 22.38$, $p < 0.01$) and 24 h (LPS main effect; $F_{1,22} = 16.00$, $p < 0.01$) after treatment. IL-1RA, which is an IL-1β-signaling antagonist, was increased (LPS main effect; $F_{1,22} = 66.24$, $p < 0.01$) in both strains in response to central LPS. However, in agreement with IL-1β data, IL-1RA remained elevated (strain x LPS interaction; $F_{3,20} = 13.10$, $p < 0.01$) only in LPS-treated WT mice at 24 h.

Consistent with reductions in proinflammatory cytokine expression, the expression of genes associated with glial activation was diminished in LPS-treated ICE KO mice compared to LPS-treated WT mice (Fig. 3.2C). CD11b expression was not influenced by LPS 4 h after treatment but was significantly lower (strain x LPS interaction; $F_{3,20} = 8.55$, $p < 0.01$) in LPS-treated ICE KO mice compared to LPS-treated WT mice at 24 h. LPS increased (LPS main effect; $F_{1,22} = 24.65$, $p < 0.01$) expression of the astrocyte activation marker GFAP at 4 h post treatment in both strains of mice. ICE KO mice tended to have lower expression (strain x LPS interaction; $F_{1,22} = 3.87$, $p = 0.06$) of GFAP at 24 h.

The similar expression of cytokines in the brains of ICE KO and WT mice at 4 h agrees with their similar sickness response. The reduced cytokine expression in the brains of ICE KO

mice compared to WT mice, at 24 h, coincides with the lack of depression-like behaviors following ICV LPS of ICE KO mice, indicating that central cytokines are involved in depression-like behaviors associated with neuroinflammation.

Systemic LPS administration induced a similar sickness response in ICE KO and WT mice.

To determine if sickness responses are similar in ICE KO and WT mice following peripheral administration of LPS, body weight was recorded immediately prior to and 24 h after treatment in both strains of mice (Table 3.2). Change in body weight was calculated and used as one index of sickness following systemic LPS challenge. Both ICE KO and WT mice treated with LPS displayed reduced (LPS main effect; $F_{1,45} = 692.01$, $p < 0.01$; Fig. 3.4A) body weight at 24 h post treatment. ICE KO and WT mice had similar sickness response following IP LPS challenge, indicating that ICE deletion does not prevent sickness response to systemic LPS. Locomotor activity was also tested in all mice 24 h post-treatment (Table 3.2). WT but not ICE KO mice treated with LPS had a reduced (strain x LPS interaction; $F_{3,43} = 10.56$, $p < 0.01$) number of line crossings and rearing in this locomotor activity test compared to saline treated controls. As observed in the central LPS studies, ICE KO mice, regardless of treatment, had fewer line crossing and rearings compared to saline treated WT mice ($p < 0.01$). ICE KO mice appear to be recovered from reductions in locomotor activity following systemic LPS, although their loss of body weight is similar.

Peripheral LPS increased FST immobility and decreased sucrose preference of both WT and ICE KO mice.

To test the hypothesis that ICE KO mice would not be protected from systemic LPS-induced depression-like behavior, we submitted both strains of mice to FST and sucrose preference test following IP administration of LPS or saline. During the FST, LPS increased

(LPS main effect; $F_{1,45} = 10.33$, $p < 0.01$) time of immobility similarly of both WT and ICE KO mice (Fig. 3.3A). To determine whether ICE KO mice developed an anhedonic response, preference for a 1% sucrose solution from 24 to 48 h post treatment was quantified (Fig. 3.3B). Both WT and ICE KO mice treated IP with LPS had reduced (LPS main effect; $F_{1,22} = 4.76$, $p < 0.05$) sucrose preference. These data confirm our hypothesis that deletion of ICE does not protect against depression-like behavior following systemic administration of LPS.

A peripheral LPS challenge increased expression of pro-inflammatory cytokines and glial activation markers similarly in the brains of both WT and ICE KO mice.

To evaluate whether LPS increased steady-state expression of mRNA for inflammatory mediators similarly in WT and ICE KO mice, qRT-PCR was used to quantify mRNA expression in brain after IP treatment with LPS or saline. Consistent with data from the central LPS studies, ICE expression was increased ($p < 0.05$) at 4 and 24 h by LPS in WT mice only. Interestingly, systemic LPS increased (LPS main effect; $F_{1,21} = 5.53$, $p < 0.05$) brain IL-18 expression in both strains at 4 h but not at 24 h ($p > 0.1$; data not shown). Systemic LPS (Fig. 3.4A) similarly increased (LPS main effect; $F_{1,21} = 127.96$, $p < 0.01$) brain expression of IL-1β in both WT and ICE KO mice at 4 h after treatment. However at 24 h, ICE KO mice had lower (strain x LPS interaction; $F_{3,19} = 6.58$, $p < 0.05$) IL-1β expression relative to LPS-treated WT mice 24 h after treatment. In contrast to central LPS-treated ICE KO mice, IL-1β remained elevated in systemic LPS-treated ICE KO mice relative to controls. Systemic LPS increased brain TNF-α (LPS main effect; $F_{1,21} = 192.89$, $p < 0.01$) and IL-6 (LPS main effect; $F_{1,21} = 41.72$, $p < 0.01$) expression at 4h. TNF-α expression remained elevated (LPS $F_{1,21} = 121.70$, $p < 0.01$) at 24 h, while IL-6 was reduced (LPS main effect; $F_{1,21} = 5.68$, $p < 0.05$) in both strains of mice. Importantly, there were no strain differences in TNF-α or IL-6 expression.

Brain expression of IL-10 and IL-1RA was measured to determine whether these transcripts were similar in ICE KO and WT mice treated with systemic LPS (Fig. 3.4B). Four hours following an IP injection of LPS, IL-10 (strain x LPS interaction; $F_{3,19} = 6.83$, $p < 0.05$) and IL-1RA (strain x LPS interaction; $F_{3,19} = 6.83$, $p < 0.05$) expression were greater in ICE KO mice compared to LPS-treated WT mice. By 24 h, both strains had similar increases (LPS main effect; $F_{1,21} = 68.83$, $p < 0.01$) in brain IL-10. However, LPS-induced IL-1RA expression was lower (strain x LPS interaction; $F_{3,19} = 7.34$, $p < 0.05$) in ICE KO than WT mice at 24 h after treatment but still remained elevated compared to control mice.

To determine if ICE influenced activation of glia in response to systemic LPS, we measured expression of microglia and astrocyte activation markers in brain (Fig. 3.4C). LPS induced CD11b (LPS main effect; $F_{1,21} = 200.97$, $p < 0.01$) expression similarly in both ICE KO and WT mice at 24 h only. Brain GFAP expression was increased similarly at 4 h (LPS main effect; $F_{1,21} = 9.06$, $p < 0.01$) and 24 h (LPS main effect; $F_{1,21} = 96.48$, $p < 0.01$) in both strains following peripheral LPS treatment. These data indicate that glial activation is similar in both ICE KO and WT following systemic LPS, unlike what was observed in ICE KO mice following central injection of LPS.

With the exception of IL-1β and IL-1RA expression, both ICE KO and WT mice have similar expression of genes in the brain that are associated with inflammation following systemic LPS. These gene expression data agree well with the induction of a similar depression-like behavior in both strains following systemic LPS.

3.5 Discussion

We previously demonstrated that ICE KO mice are resistant to central LPS-induced reduction in food intake (Yao et al., 1999) and feeding behavior (Burgess et al., 1998). However,

ICE KO mice lack this protection following systemic LPS administration (Burgess et al., 1998) although they are protected against peripheral endotoxic shock induced by higher doses of LPS (Li et al., 1995). These findings suggested that there is a distinction between responses elicited by an activation of the central innate immune system versus the peripheral immune system. Given the more recent data showing that IL-1 is critically involved in the development of depression-like behaviors (Craft and DeVries, 2006; Goshen et al., 2008; Koo and Duman, 2008; Norman et al., 2010), these findings stimulated us to test the hypothesis that ICE KO mice might be protected from central but not peripheral LPS-induced depression-like behaviors. To our knowledge, we are now the first to report that ICE is required for development of depression-like behavior following a central, but not systemic, LPS challenge.

ICE is a cysteine protease and is the enzyme principally responsible for cleavage of two critical pro-inflammatory cytokines, pro-IL-1β and pro-IL18, from their inactive precursors to their mature active secreted forms. ICE is constitutively present within cells as an inactive precursor colocalizing with a group of proteins that collectively form the inflammasome. Inflammasomes contain nucleotide and oligomerization domain-like receptor (NLR) family proteins that act as intracellular receptors for pathogen associated molecular patterns (PAMPs) or danger associated molecular patterns (DAMPs) in a similar fashion as the widely recognized toll-like receptors (Lamkanfi and Dixit, 2009; Fleshner, 2012; Iwata et al., 2012). Following recognition of PAMPs and DAMPs by NLRs, there is a transient increase in ICE activity (Franchi et al., 2009). The precise mechanism by which LPS can directly activate inflammasomes and ICE activity has not been fully elucidated. However, LPS-induced upregulation of ICE expression has largely been shown to occur in cells of the myeloid lineage such as central microglia (Yao and Johnson, 1997) and peripheral monocytes/macrophages (Li et

al., 1997). We confirmed that ICE expression in the brain was upregulated by both central and peripheral LPS in WT mice.

ICE activity has substantial influence over behavioral responses during inflammation, presumably through its role in processing inactive IL-1β (Franchi et al., 2009). Genetic deletion of ICE decreases LPS-induced IL-1β secretion (Li et al., 1995). It has been well established that IL-1β activates the hypothalamic-pituitary-adrenal axis and induces behavioral changes associated with sickness and depression, such as anhedonia, disruption of sleep, cognitive disturbances, temperature disregulation and consumption of food (Besedovsky and del Rey, 2000; Rothwell and Luheshi, 2000; Goshen and Yirmiya, 2009). Our findings indicate a prominent role for central ICE during inflammation-induced depression. These new data are in in agreement with recent work that has demonstrated a role for IL-1ß in the development of depression-like behavior utilizing a variety of models. Chronic exposure to IL-1β diminished sucrose preference and social exploration; which are indicative of the depressive symptoms of anhedonia and social withdrawal, respectively (Goshen et al., 2008; Nestler and Hyman, 2010). Chronic mild stress (CMS) of mice not only elevates IL-1β levels but also results in depression-like symptoms, including a decrease in sucrose preference (to the point of aversion) and decreased social exploration. These symptoms are dependent on IL-1β as either type 1 IL-1 receptor (IL-1R) deficiency or the injection of IL-1 receptor antagonist (IL-1RA) block the effect of CMS (Goshen et al., 2008). Similarly, several other studies using models of CMS, chronic unpredictable stress (CUS), chronic pain and ischemic stroke have demonstrated that reducing IL-1 signaling blocks depression-like behaviors including reduced sucrose preference and increased immobility time in the FST (Craft and DeVries, 2006; Koo and Duman, 2008; Norman et al., 2010). Similar CUS causes mice to display decreased preference for sucrose solution

compared to unstressed mice, and this anhedonic response is blocked by ICV administration of IL-1RA (Koo and Duman, 2008). This later finding is important as it illustrates that IL-1β activity within the brain is required for the development of depression-like behavior. Chronic pain has also been linked to depression with a possible cause and effect relationship (Fishbain et al., 1997; Lepine and Briley, 2004). Moreover, IL-1 has been extensively examined for its role in mediating symptoms of neuropathic pain (reviewed in (Marchand et al., 2005)). Utilizing the spared nerve injury model of chronic pain, increased time of immobility during the FST was blocked by central administration of IL-1RA into the lateral ventricle. Middle cerebral artery occlusion, which is a model of ischemic stroke, leads to a robust induction of brain IL-1β expression and IL-1 signaling in this model plays an important role in post-stroke depression as evidenced by a reduction in sucrose consumption that is blocked when mice are treated with an ICV injection of IL-1RA (Craft and DeVries, 2006). All of these studies established that brain IL-1 signaling is of significant importance for mediating depression-like behaviors since each model targeted IL-1 signaling in the brain. All of these results are in agreement with the new findings reported here that deletion of ICE blocks the LPS-induced increase in FST immobility and decrease in sucrose preference only when LPS is administered centrally. Clearly, non-ICE dependent mechanisms for depression-like behavior remain functional when LPS is administered peripherally.

Despite reduced IL-1β secretion, ICE KO mice have similar a sickness response compared to WT mice in terms of loss of body weight following both systemic and central LPS challenges. This lack of attenuation of the sickness response is not surprising since IL-1R1 knockout mice also lose body weight similar to WT mice when treated with ICV or IP LPS (Bluthe et al., 2000). Further, inhibiting IL-1 signaling with ICV or IP administration of IL-1RA

is not sufficient to block body weight loss in LPS treated rats (Bluthe et al., 1992). It was postulated several years ago that LPS-induced sickness behaviors require the presence of either TNF-α or IL-1β, but when IL-1 signaling is absent, TNF-α assumes a more prominent role (Bluthe et al., 2000). This postulate is consistent with the present findings that both WT and ICE KO mice displayed elevated TNF-α expression in the brain following LPS. Therefore, similar degrees of sickness behavior following LPS injection in WT and ICE KO mice is consistent with the known pleiotropic properties of both IL-1β and TNF-α.

Unexpectedly, ICE KO mice had reduced locomotor activity as assessed by the number of line crossings and rearings compared to WT mice. A possible explanation for reduced exploratory behavior measured in ICE KO mice is increased anxiety in these mice. Exploratory behavior is often used as an important screening tool for anxiety-like behavior (Crawley, 1985). We cannot rule out the possibility that reduced locomotor activity exhibited by ICE KO mice is indicative of an anxiogenic phenotype but our experiments were not designed to properly test this possibility. However, it is important to note that locomotor activity of ICE KO mice 24 h after LPS was unaffected by either central or systemic LPS challenge. We interpret these data to suggest that ICE KO mice display a more rapid recovery from sickness behavior compared to WT mice. Again, testing this possibility was not the focus of our experiments. Importantly for the current body of work, the decrease in locomotor activity did not translate into an elevated time of immobility during the FST. The equal time of immobility of saline treated WT and ICE KO mice during this test indicates that reduced locomotor activity was not due to depression-like behavior and that differences in performance during the FST did not result from a motor deficit. However, as ICE inhibitors may draw increasing interest as treatments for inflammation-

associated diseases, it will be important to evaluate different alterations for other ICE-dependent behaviors.

ICE KO mice have reduced IL-1β secretion in response to LPS (Li et al., 1995) but still develop depression-like behavior following systemic but not central LPS administration. This finding alludes to compensatory actions of other peripheral cytokines to induce inflammatory mediators within brain in the absence of elevated IL-1β or a lack of peripheral IL-1ß involvement in peripherally induced depression-like behaviors. In our experiments, both WT and ICE KO mice displayed increased early (4 h) expression of brain IL-1β, TNF-α and IL-6 following both systemic and central LPS. At 24 h, brain IL-1β and TNF-α were no longer elevated in central LPS-treated ICE KO compared to saline-treated ICE KO mice even though expression of these genes remained elevated in LPS-treated WT mice. In our model, the reduced induction of IL-1β mRNA expression following LPS treatment likely results from a quicker extinguishing of the feed-forward cytokine signaling, in agreement with data demonstrating that IL-1β induces its own expression and the expression of TNF-α and IL-6 within brain (Depino et al., 2005; Taishi et al., 2008). This was in contrast to what was observed following systemic LPS because most inflammatory mediators that we measured were increased similarly in WT and ICE KO mice. In contrast, TNF-α expression remained elevated at 24 h following systemic LPS administration in both WT and ICE KO mice. This finding indicates that even in the absence of IL-1ß secretion, TNF-α may mediate depression-like behavior because its absence in the brain at 24 h in ICV-treated ICE KO mice corresponds to a lack of depression-like behavior and its continued presence at 24 h in ICV-LPS WT, IP-LPS WT and IP-LPS ICE KO mice corresponds to the presence of depression-like behaviors. A role for TNF-α in depression-like behavior has been directly shown. Even extremely low doses of TNF-α administered ICV causes depression-

like behavior as assessed as increased time of immobility during both the FST and tail suspension test (Kaster et al., 2012). In addition, TNF-R1 deficient mice and mice treated with a neutralizing antibody to TNF-α had a decreased time of immobility during the FST, indicating an anti-depressant response. This study supports work showing that TNF receptor deficient mice have lower immobility during the FST, again indicating an anti-depressant response. The TNF receptor deficient mice also have increased consumption of a sucrose solution, indicative of an anhedonic response mediated by TNF-α (Simen et al., 2006). In further support of a role for TNF-α in depression, human patients afflicted with plaque psoriasis showed significant improvement in Beck Depression Inventory and Hamilton Rating Score for depression when treated with the TNF neutralizing drug Etanercept (Tyring et al., 2006). Patients treated with Etanercept showed significant improvement in sexual function, sleep, irritability and other symptoms of depression that impacted quality of life compared to patients receiving placebo. These data indicate that TNF-α is probably involved in mediating depression-like behaviors. Together with our current data, we hypothesize that in the absence of IL-1ß, depression-like behavior is present only when central TNF-α expression is elevated following the LPS challenge.

Expression of IL-10 and IL-1RA in the brain remain elevated in ICE KO and WT mice following systemic LPS exposure at 24 h. This was not the case following central LPS, further supporting a role for IL-1β in a sustained brain inflammatory response. Compensatory actions of other cytokines such as TNF-α (McCusker and Kelley, 2013) contribute to peripheral immune activation cascades when IL-1 action is lost. We also found that ICE KO mice have decreased mRNA expression of genes associated with microglia and astrocyte activation, CD11b and GFAP, following central LPS treatment. We interpret these findings as evidence that IL-1β is important for maintaining activation of glial cells in response to neuroinflammation. Indeed,

reduced cytokine expression observed in ICE KO following central LPS is reflective of reduced glial cell activation. We are intrigued by our finding that brain MHCII expression was not different between ICE KO and WT mice following central LPS (data not shown) as this may indicate a less prominent role for IL-1β to induce an antigen presenting phenotype in microglia. Based on these data, our results add to evidence that ICE and subsequently IL-1ß signaling plays a necessary role for initiating and sustaining a full inflammatory response within the brain that manifests behaviors associated with depression. To our knowledge, we are first to report that ICE KO mice are protected from central LPS-induced depression-like behavior. Deletion of ICE has a significant impact on the inflammatory profile in brain following ICV LPS but essentially no effect on the brain following a peripheral LPS challenge. We propose that targeting of ICE represents a potential therapeutic target directed at treating neuroinflammation-dependent comorbid depression.

3.6 Figures

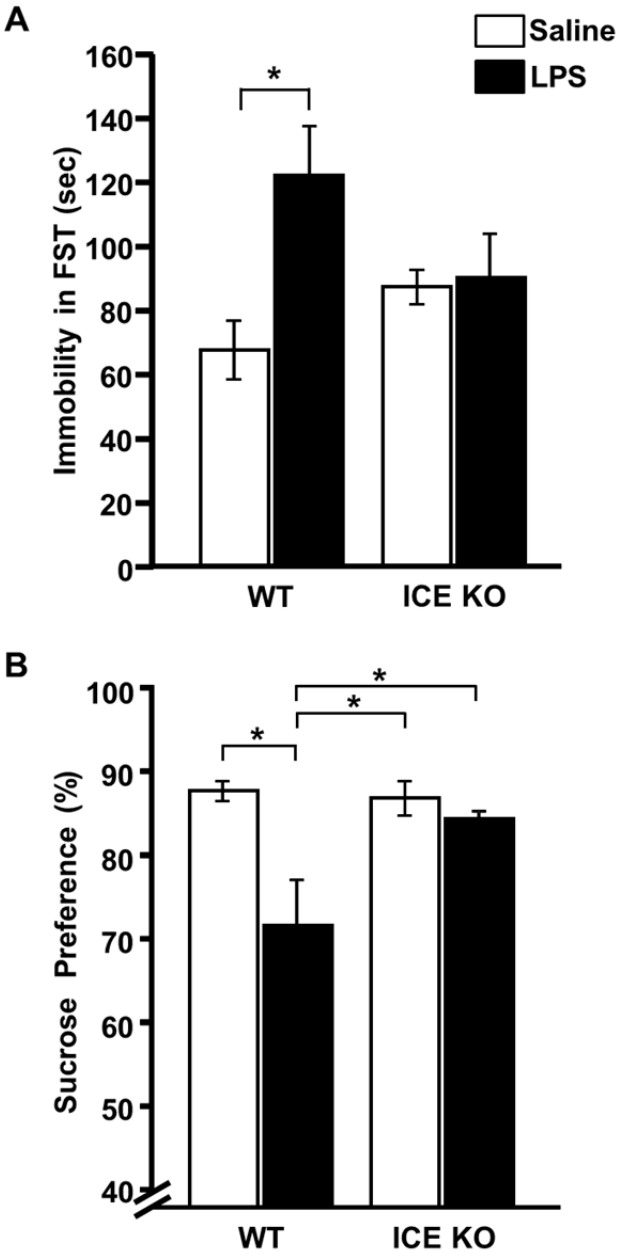

Figure 3.1: ICE KO mice were protected from depression-like behaviors following ICV administration of LPS compared to WT mice. A) Time of immobility in the FST was determined 24 h after treatment. WT mice given LPS had greater time spent immobile compared to control WT mice, while immobility time in LPS-treated ICE KO mice was not different from control mice. B) WT, but not ICE KO, mice displayed decreased preference for sucrose during the 24 – 48 h period following LPS injection. Data represent averages ± SEM. n = 9-12 mice per group, * $p < 0.05$ comparing bracketed treatment groups.

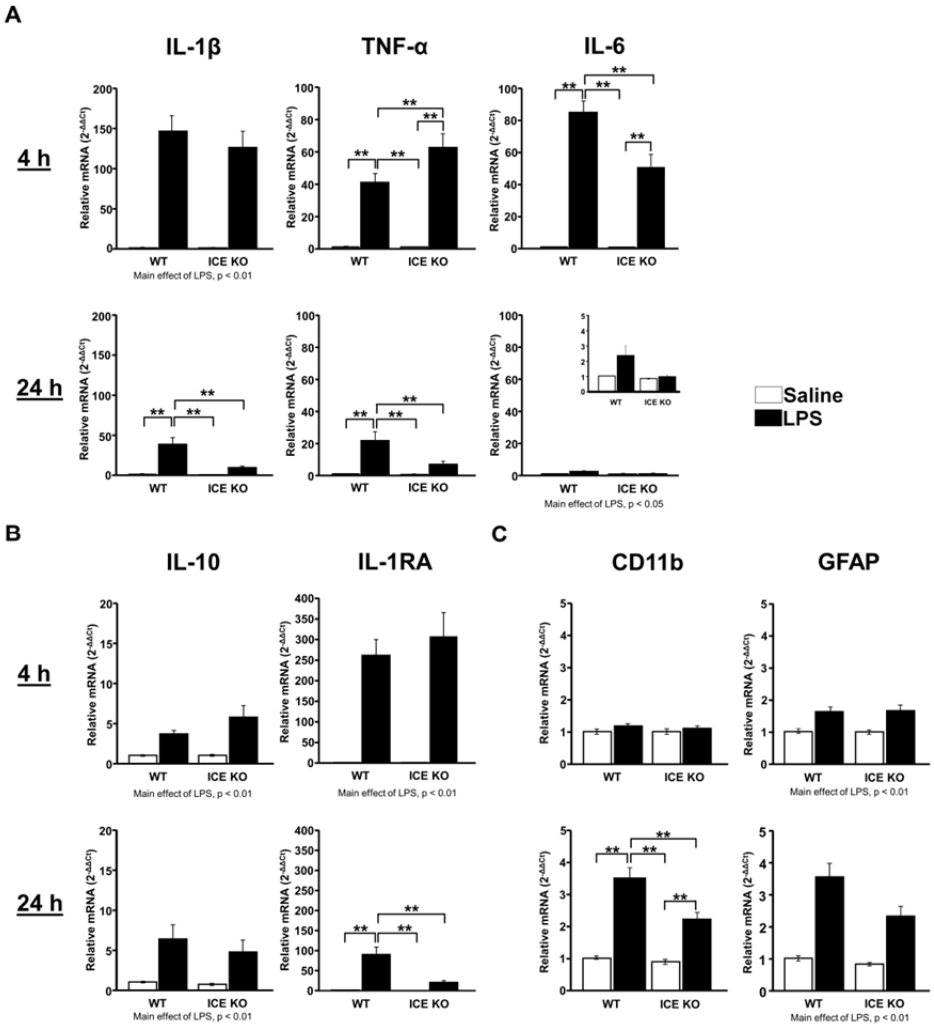

Figure 3.2: Expression of proinflammatory cytokines in brain was reduced in ICE KO mice at 24 h following LPS given ICV. A) LPS increased mRNA expression of IL-1β, TNF-α and IL-6 in WT and ICE KO at 4 h. Expression of IL-1β and TNF-α remained elevated in brains of only WT mice at 24 h. B) IL-10 expression was increased similarly by central LPS in both strains of mice at 4 h and 24 h. In contrast, IL-1RA mRNA expression increased in both strains at 4h but remained elevated at 24 h only in WT mice. C) CD11b and GFAP expression were increased similarly in WT and ICE KO brains at 4 h. At 24 h, CD11b expression was reduced in ICE KO mice compared to WT mouse brains following ICV administration of LPS. Data are average mRNA expression levels relative to GAPDH ± SEM; ** $p < 0.01$, * $p < 0.05$ comparing bracketed groups; main effects of LPS where indicated; n = 6 mice per group. Insets: Data presented with zoomed in scale to ease interpretation. Mean Ct values for saline treated WT groups were: IL-1β, 31.7 ± 0.4; TNF-α, 32.0 ± 0.3; IL-6, 31.6 ± 0.4; IL-10, 34.6 ± 0.2; IL-1RA, 33.2 ± 0.3; CD11b, 20.7 ± 0.03; GFAP, 17.4 ± 0.1.

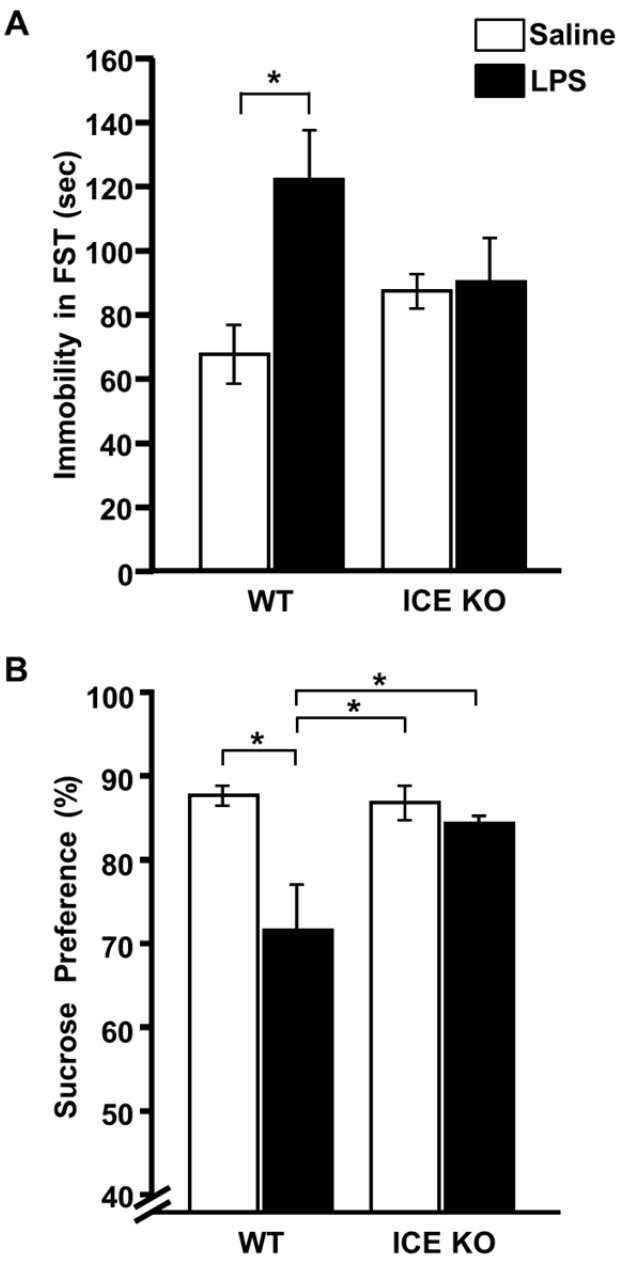

Figure 3.3: Both ICE KO and WT mice displayed depression-like behaviors following IP LPS challenge. LPS given systemically increased (A) immobility in the FST 24 h after treatment and decreased B) sucrose preference when measured 24 – 48 h post-treatment similarly in both mouse strains. Data represent averages ± SEM; main effects of LPS where indicated; n = 6 mice per group.

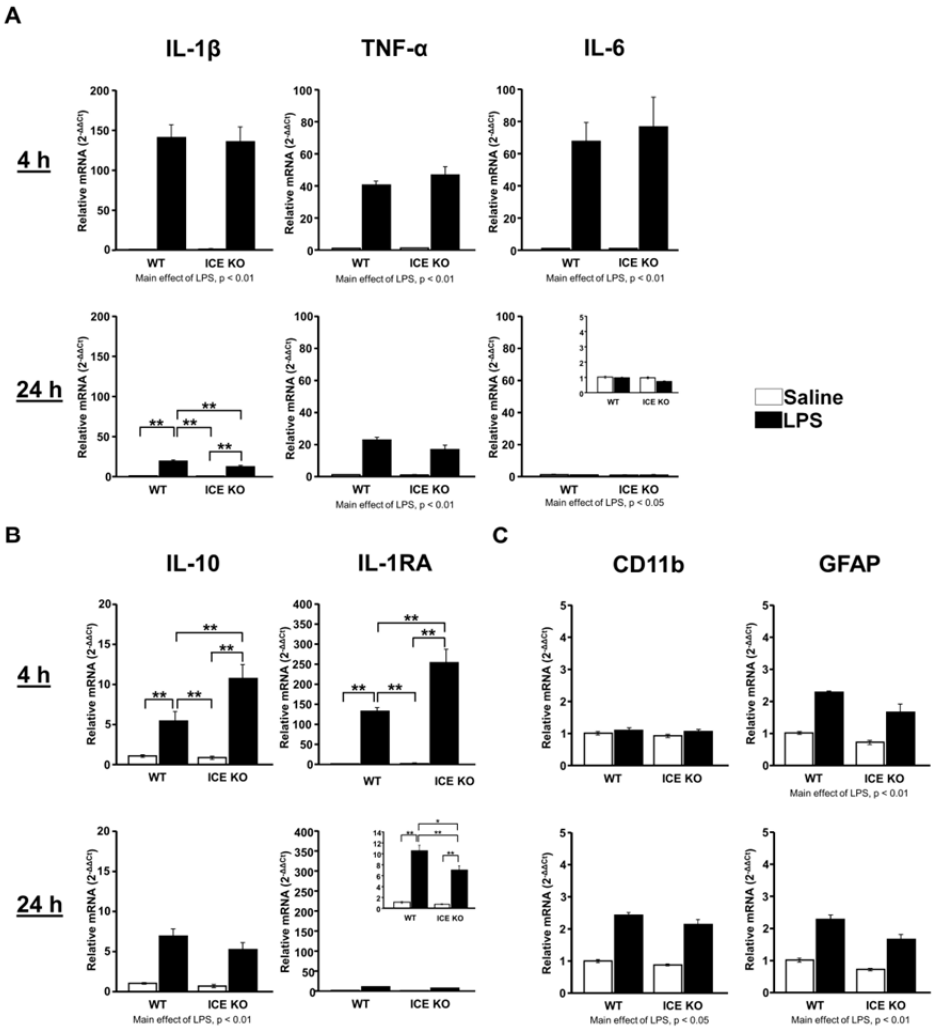

Figure 3.4: Both ICE KO and WT mice given systemic LPS exhibited increased expression of inflammatory markers in brain. In both WT and ICE KO mice, LPS injected IP induced expression of A) IL-1β, TNF-α and IL-6 at 4 h. All but IL-6 remained elevated at 24 h. B) ICE KO mice had greater expression of IL-10 and IL-1RA at 4h. However, at 24 h, the LPS-induced increase in IL-10 expression was similar in both strains of mice while that of IL-1RA was reduced in ICE KO mice. C) CD11b was not elevated by LPS until 24 h post-treatment while GFAP expression was increased at both 4 h and 24 h similarly in WT and ICE KO mice. Data are average mRNA expression levels relative to GAPDH ± SEM, ** $p < 0.01$, * $p < 0.05$ comparing bracketed groups; main effects of LPS where indicated; n = 5-6 mice per group. Insets: Data presented with zoomed in scale to ease interpretation. Mean Ct values for saline treated WT groups were: IL-1β, 29.5 ± 0.1; TNF-α, 29.6 ± 0.1; IL-6, 29.2 ± 0.1; IL-10, 34.9 ±0.1; IL-1RA, 30.4 ± 0.3; CD11b, 21.6 ± 0.1; GFAP, 18.0 ± 0.1.

3.7 Tables

Table 3.1. Sickness Response Measures Following ICV LPS

	Δ Body Weight (g / 24 h)	Line Crossings (# / 5 min)	Rearings (# / 5 min)
WT	-0.10 ± 0.12^{a}	62.7 ± 5.5^{a}	50.3 ± 5.0^{a}
WT-LPS	-1.83 ± 0.39^{b}	63.3 ± 6.5^{a}	47.9 ± 5.0^{a}
ICE KO	0.17 ± 0.13^{a}	30.0 ± 2.6^{b}	25.7 ± 1.9^{b}
ICE KO-LPS	-1.64 ± 0.12^{b}	35.5 ± 6.4^{b}	28.5 ± 5.2^{b}

Body weight was measured at 0 and 24 h and body weight change was calculated from these measures; locomotor activity was tested 24 h after treatment. Averages within columns with different letters are significantly different; $p < 0.05$.

Table 3.2. Sickness Response Measures Following IP LPS

	Δ Body Weight (g / 24 h)	Line Crossings (# / 5 min)	Rearings (# / 5 min)
WT	-0.02 ± 0.11^{a}	60.3 ± 5.6^{a}	42.9 ± 5.0^{a}
WT-LPS	-2.84 ± 0.09^{b}	36.8 ± 6.5^{b}	26.9 ± 3.0^{b}
ICE KO	-0.09 ± 0.10^{a}	35.6 ± 3.8^{b}	27.0 ± 3.0^{b}
ICE KO-LPS	-2.82 ± 0.12^{b}	36.9 ± 2.6^{b}	25.2 ± 2.4^{b}

Body weight was measured at 0 and 24 h and body weight change was calculated from these measures; locomotor activity was tested 24 h after treatment. Averages within columns with different letters are significantly different; $p < 0.05$.

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Chapter 4

Intracerebroventricular administration of lipopolysaccharide induces indoleamine 2,3 dioxygenase-dependent depression-like behaviors

4.1 Abstract

Activation of the tryptophan degrading enzyme indoleamine 2, 3 dioxygenase 1 (IDO1) is associated with the development of behavioral signs of depression. Systemic immune challenge induces IDO1 in both the periphery and brain leading to increased circulating and brain concentrations of kynurenines. However, it remains to be elucidated whether IDO1 activity within the brain is necessary for the manifestation of depression-like behavior of mice following a central immune challenge. Here, we investigated the role of brain IDO1 in mediating depression-like behavior of mice in response to intracerebroventricular (ICV) injection of Saline or lipopolysaccharide (LPS, 10 ng). LPS increased the duration of immobility in the tail suspension test (TST) and decreased preference for a sucrose solution. These effects were associated with an activation of central but not peripheral IDO1, as LPS increased brain kynurenine but had no effect on plasma concentrations of kynurenine. Interestingly, genetic deletion or pharmacological inhibition of IDO1, using 1-methyl-tryptophan (1-MT), abrogated the reduction in sucrose preference induced by icv LPS.. 1-MT also blocked the LPS-induced increase in duration of immobility during the TST. These data indicate that activation of brain IDO1 is required to induce depression-like behaviors of mice in response to central LPS.

4.2 Introduction

Over the past several decades, a link between inflammation and neuropsychiatric disorders has been firmly established at both clinical and preclinical levels (Miller et al., 2009). In depressed patients, many studies have reported elevated levels of peripheral inflammatory

markers, e.g. plasma IL-6 and C-reactive protein associated with symptoms of major depression (Maes et al., 1997; Howren et al., 2009). Also, peripheral inflammation induced by lipopolysaccharide (LPS), in both human volunteers and rodent models, precipitates significant changes in cognitive function, mood and behavior (Yirmiya, 1996; Reichenberg et al., 2001; Krabbe et al., 2005; O'Connor et al., 2009a). Individuals afflicted with neurodegenerative diseases that have a neuroinflammatory signature such as multiple sclerosis (Feinstein, 2011), Huntington's disease (Rosenblatt, 2007), Parkinson's disease (Aarsland et al., 2012) and Alzheimer's disease (Aznar and Knudsen, 2011) have increased prevalence of comorbid neuropsychiatric disturbances. Principal in these processes is the increased expression of pro-inflammatory cytokines in the brain that mediate the core neuropsychiatric and neurovegetative symptoms of major depression (Dantzer et al., 2008).

Cytokines released within the brain can directly impact neuron function, as evidenced by IL-1ß- and TNFα-induced changes in long-term potentiation (Cunningham et al., 1996; O'Connor and Coogan, 1999; Cumiskey et al., 2007), but they can also act indirectly by stimulating the production of neuroactive molecules that have been associated with depression. In the last decade, increasing interest has focused on the tryptophan degrading enzyme indoleamine 2,3-dioxygenase 1 (IDO1). During inflammation, IDO1 is the first and rate limiting enzyme in a metabolic cascade that leads to increased levels of kynurenine in the circulation and tissues. Moreover, increased IDO1 enzymatic activity and elevated kynurenine concentration levels have been correlated with inflammation-associated depression (Christmas et al., 2011; Dantzer et al., 2011; Myint, 2012; Schwarcz et al., 2012). Recent preclinical research has demonstrated that pharmacological inhibition of IDO1 enzymatic activity or genetic deletion of IDO1 abrogates inflammation-dependent behavioral changes that model depression. This has

been demonstrated in a murine model of acute inflammation induced by peripheral LPS and in a model of chronic inflammation induced by peripheral infection with Bacille Calmette Guérin (BCG) (O'Connor et al., 2009b; O'Connor et al., 2009c; O'Connor et al., 2009a; Salazar et al., 2012). There is already evidence that intracerebral activation of cytokine signaling pathways by intracerebroventricular administration of TNF-α, LPS or the human immunodeficiency virus transactivator of transcription (Tat) precipitates the development of depression-like behavior in rodents that is associated with upregulation of cytokines and IDO1 (Lawson et al., 2011; Dobos et al., 2012; Kaster et al., 2012). However, the causal role of brain IDO1 activation in these models has not yet been established.

In order to explore the potential for brain kynurenine metabolism to drive inflammation-induced depression-like behavior, we challenged mice with a single intracerebroventricular (ICV) injection of LPS. Central LPS induced depression-like behaviors coupled with increased kynurenine concentrations and kynurenine:tryptophan ratio specifically within the brain. In contrast, IDO1 knockout mice and wild-type mice pretreated with 1-MT were protected from developing LPS-induced depression-like behavior. Taken together, these data indicate that upregulation of brain IDO1 is required for the development of depression-like behaviors following ICV LPS.

4.3 Materials and Methods

Animals

Male Balb/C mice (Charles Rivers Laboratories, Wilmington, MA), C57BL/6J (WT) mice and IDO1 KO mice (The Jackson Laboratory, Bar Harbor ME) were individually housed and provided with ad libitum access to chow (Teklad 8640) and water. Mice were housed in a temperature and humidity controlled room maintained on a 12 hour reversed light/dark cycle.

Mice were allowed to acclimate to these conditions for at least 7 days before being implanted with a guide cannula for mice (Plastics One, Roanoke, VA) placed stereotaxically to extend 1mm dorsal to the lateral ventricle as previously described (Lawson et al., 2011). Cannulas were placed at 1.5 mm lateral, 0.6 mm posterior, and 1.3 mm dorsal with respect to bregma (Fanklin, 2001). Guide cannulas were kept clean and covered using a screw-on dummy cannula (Plastics One, Roanoke, VA). Mice were given 10-14 days to recover from surgery prior to treatment. All procedures performed on mice were in compliance with the National Institutes of Health guidelines and approved by the Institutional Animal Care and Use Committees at both the University of Texas Health Science Center at San Antonio and the University of Illinois at Urbana-Champaign.

Treatments

Treatments were administered at the onset of the dark cycle. ICV injections were administered using a 10 µl gas-tight syringe attached to internal injector cannulas (Plastics One, Roanoke, VA) that extended 1 mm beyond the tip of the guide cannula, thus penetrating the lateral ventricle. All mice received treatments in 1 µL injection volume over a one minute time period followed by an additional one minute delay to allow diffusion before removing injector cannula. Mice were injected ICV with either phosphate buffered saline (saline) or with LPS (10 ng/µL) from Escherichia coli O127:B8 (Sigma Aldrich, St. Louis, MO) prepared in saline.

In experiments examining the effects of 1-MT (1-methyl-DL-trypophan, 1 µg/µL in injectable saline (pH 6.5), Sigma Aldrich, St. Louis, MO), WT mice were treated in 2 x 2 factorial arrangement with an ICV injection of saline, 1-MT, LPS or 1-MT+LPS. The treatments were combined so that mice were given a single 1 µL ICV injection. 1-MT was prepared by mixing with 1 N HCl, then buffered using sodium hydroxide to pH 6.5 before being diluted to

the final treatment concentration. The dose of 1-MT used was based on the average brain 1-MT concentrations in mice implanted with a subcutaneous chronic release pellet (O'Connor et al., 2009a; Corona et al., 2012).

Depression-like Behavior

Two tests of depression-like behavior were utilized in each study (Nestler and Hyman, 2010). At 24 h after treatment, mice were submitted to the TST using the Mouse Tail Suspension Package (Med Associates, St Albans, VT) as previously described (Park et al., 2011b). During the TST, mice were suspended by their tail using adhesive medical tape attached to a strain force gauge. Mice were tested for 10 min and were considered immobile when the force was below a lower threshold that was determined for each individual mouse.

To determine whether mice display signs of anhedonic behavior, we utilized a two-bottle sucrose preference test. Mice were given ad libitum access to drink from bottles containing either water, 1% (C57BL6/J) or 2% (Balb/C) sucrose solution for a 24 h period. Immediately prior to being placed on the home cage (immediately after treatment) and 24 h later the bottles were weighed so that the amount of each solution that was consumed could be calculated. Preference was calculated by determining the percent of sucrose consumed divided by the total fluid intake (Sucrose Intake/ Total Fluid Intake * 100).

Tissue Collection and Processing

After behavioral testing, mice were rapidly euthanized in a CO2 chamber and laparotomized to facilitate blood collection and vascular perfusion. Blood samples were collected from the inferior vena cava. Immediately following blood collection the chest was opened, a knick was made in the right atrium and ~30 ml ice-cold PBS was perfused via the left ventricle. Brains were then rapidly removed and placed in a vial on dry ice. The brains were stored at -

80°C until processing. Brains were removed from -80°C storage and pulverized with a ceramic mortar and pestle chilled on dry ice to maintain the brains in a frozen state. The brain powder was mixed to homogenize the tissue and the powder was then aliquoted for analysis. The purpose of this procedure was to divide the tissue equally and negate potential effects of hemispheric differences brought about by cannulation.

HPLC Methods

Homogenized brain and plasma samples were analyzed for kynurenine and tryptophan using a Coulochem III electrochemical detector paired with a model 5041 amperometric analytical cell fitted with a glassy carbon target electrode (Thermo Scientific Dionex, Bannockburn, IL). Mobile phase (pH 4.6) consisted of 75 mM NaH2PO4, 25 μM EDTA, 100 μl/L triethylamine mixed into acetonitrile:water (6:94; v:v). Compounds of interest were separated with a Hypersil ODS C18 anlytical column (2.1 mm x 150 mm; 3μM; Thermo Scientific, West Palm Beach, FL) and compared against an external standard curve made fresh on each day of analysis. Chromatograms were collected and analyzed using EZ Chrom SI software (Agilent Technologies, Santa Clara, CA).

Statistical Analysis

Data are represented as the means ± SEM. All measures were analyzed using a one-way analysis of variance (ANOVA) or a two-way ANOVA in IDO KO and 1-MT studies. When the two-way interaction was significant ($p<0.05$), post-hoc analysis using Fishers protected least significant difference test was employed to test for differences among means.

4.4 Results

Intracerebroventricular administration of LPS precipitates the development of depression-like behaviors in mice.

To confirm that central LPS induced a sickness response, we evaluated change in body weight. As expected, Balb/c mice injected ICV with 10 ng LPS lost body weight over 24 h period following treatment ($F_{1,12} = 5.63$, $p < 0.05$, Fig. 4.1A). We further evaluated whether central LPS induced depression-like behaviors. Sucrose preference was measured during the 24 h period following treatment. LPS treated mice displayed a significant decrease in sucrose preference ($F_{1,12} = 8.31$; $p < 0.05$, Fig. 4.1B). Mice were submitted to TST 24 h after treatment. LPS caused a significant increase in the duration of immobility when compared to saline treated control mice ($F_{1,12} = 16.18$; $p < 0.01$, Fig. 4.1C).

Kynurenine to Tryptophan ratio is increased in brain, but not plasma, following ICV LPS treatment.

To determine if LPS-induced depression-like behavior was associated with elevated kynurenine and ratio of kynurenine to tryptophan, plasma and whole brain samples were collected and analyzed. LPS-treated mice had elevated brain kynurenine concentrations compared to saline treated mice ($F_{1,11} = 10.24$, $p < 0.01$, Table 4.1), but there was no effect of LPS on brain tryptophan concentrations. Further, elevated brain kynurenine led to an increase in the kynurenine to tryptophan ratio ($F_{1,11} = 11.26$, $p < 0.01$). In contrast, LPS treatment had no effect on plasma kynurenine concentration, tryptophan concentration or kynurenine/tryptophan ratio compared to plasma from saline treated mice (Table 1).

IDO1 knockout mice maintain sucrose preference following ICV LPS.

To determine if IDO1 activity is required for central LPS to induce depression-like behavior, we examined whether IDO1 KO mice develop depression-like behavior. Similar to WT mice, IDO1 KO mice experienced a reduction in body weight following ICV LPS treatment (LPS main effect; $F_{1,45} = 19.7$, $p < 0.01$, Fig. 4.2A). However, while LPS precipitated a significant 25% reduction in sucrose preference in wild-type mice, IDO1 KO mice were entirely refractory to the anhedonic effects of ICV LPS, compared to controls (Strain x LPS interaction; $F_{3,44} = 9.18$, $p < 0.01$, Fig. 4.2B). In contrast, IDO1 KO mice were not protected from the LPS-induced increase in immobility during the TST (LPS main effect; $F_{1,22} = 18.29$, $p < 0.01$, Fig 4.2C). These data indicate that IDO1 KO mice exhibit a similar behavioral response to LPS as WT mice during the TST. However, IDO1 KO mice do not display reduced preference for sucrose solution implicating brain IDO1 as a critical mediator of the anhedonic response following ICV LPS.

Central 1-MT treatment protects mice from central LPS-induced depression-like behavior.

To further test our hypothesis that brain IDO1 activity is necessary for developing depression-like behavior in response to ICV LPS, mice were treated with ICV saline or 1-MT concurrently with or without LPS. LPS reduced body weight 24 h following treatment in both saline or 1-MT co-treated mice (LPS main effect; $F_{1,21} = 29.39$, $p < 0.01$, Fig. 4.3A) indicating that 1-MT does not block the non-specific sickness response. Treatment with 1-MT significantly attenuated the anhedonic effects of LPS (1-MT x LPS interaction: $F_{3,19} = 5.18$, $p < 0.05$, Fig. 4.3B) although LPS decreased preference for sucrose solution of both saline and 1-MT treated mice (LPS main effect; $F_{1,21} = 26.48$, $p < 0.01$, Fig. 4.3B). As expected, LPS increased the duration of immobility in the TST in saline treated control mice (Fig. 4.3C). However, the

duration of immobility was unchanged following LPS in mice co-treated with 1-MT (1-MT x LPS interaction; $F_{3,19} = 6.43$, $p < 0.05$). Interestingly, mice that were only treated with 1-MT also exhibited reduced TST immobility compared to control + saline or control + LPS treated mice ($p < 0.01$). Taken together, these data indicate that inhibiting IDO1 with 1-MT does not protect mice from the non-specific sickness response; however 1-MT protected mice from the development of depression-like behavior following ICV LPS. These data support the hypothesis that IDO1 in brain is necessary for inducing depression-like behavior following ICV LPS.

4.5 Discussion

Here we show that ICV LPS induces depression-like behavior that corresponds to increased brain kynurenine concentrations. We have further implicated that brain IDO1 activity is necessary for depression-like behavior following ICV LPS, since genetic deletion or pharmacological inhibition of brain IDO1 protects mice from LPS-induced depression-like behavior.

Previous studies established 10 ng of LPS as an ICV dose sufficient to induce central IDO1 expression and transient sickness followed by detectable depression-like behaviors (Park et al., 2011b). Importantly, we have now demonstrated that this dose of LPS increases IDO1 activity specifically in the brain, as there were no measurable changes in plasma concentrations of kynurenine or tryptophan. Further, LPS administered ICV induced depression-like behavior concurrent with elevations in brain kynurenine, and IDO1 was required for this behavioral response. Our previous work has demonstrated that IDO1 activation and subsequently increased kynurenine is necessary for inflammation-induced depression-like behavior following systemic immune challenge (O'Connor et al., 2009c; O'Connor et al., 2009a; Salazar et al., 2012). We have also shown that increasing circulating kynurenine via exogenous administration of

kynurenine to naïve mice is sufficient to induce depression-like behavior (O'Connor et al., 2009a; Salazar et al., 2012). However the respective role of peripheral and brain IDO1 in inflammation-induced depressive-like behavior was not assessed in these studies.

To determine whether activation of brain IDO1 is required for depression-like behavior, we selected a dose of LPS that when administered ICV has no measured effect on peripheral IDO1. We tested the behavioral effect of this dose of LPS in WT mice and in mice whose IDO1 activation was blocked genetically or pharmacologically. In a manner similar to what we previously observed following systemic LPS, ICV LPS elicited a non-specific sickness response in mice whose IDO1 activation was blocked, as evidenced by body weight loss. However, a different picture emerged when assessing depression-like behavior. IDO1 KO mice maintained sucrose preference following ICV LPS; whereas, WT mice exhibit diminished sucrose preference. In contrast, IDO1 KO mice were not protected from the LPS-induced increase in immobility in the TST, although saline treated IDO1 KO mice tended to have reduced TST immobility compared to WT mice. The lack of protection observed in IDO1 KO mice during TST testing would indicate that this behavior may be influenced by the induction of other inflammatory mediators acting independently of IDO1 activation.

Convincing evidence exists that cytokines can influence depression-like behaviors independent of IDO1 expression. Our previous research examining the protective effects of insulin-like growth factor-I (IGF-I) demonstrated that ICV IGF-I, administered prior to LPS, protected against LPS-induced increase in TST immobility (Park et al., 2011b). However when the potential protective benefit of IGF-I was investigated in the sucrose preference test, mice administered IGF-I prior to an LPS challenge were not protected against LPS-induced anhedonia (Park et al., 2011a). In these studies, IGF-I decreased central proinflammatory cytokine

expression but did not attenuate the induction of brain IDO1 expression. Noteworthy is that we have previously demonstrated that cytokine expression in IDO1 KO mice is not different from WT mice following LPS (O'Connor et al., 2009c). These findings suggest that elevated cytokine expression may be necessary to precipitate depression-like behavior in the TST in response to direct neuroimmune challenge, and indeed may be adequate for this response in the absence of IDO1. However, activation of the kynurenine pathway may be necessary for central LPS to induce the anhedonic response.

An interesting paradox emerged in the present dataset; complete genetic deletion of IDO1 protected mice from the behavioral effects of LPS only in the sucrose preference test. Meanwhile, administration of 1-MT elicited an antidepressant effect in LPS treated mice in both the sucrose preference test and the TST. While still speculative, compensatory expression of indoleamine 2,3-dioxygenase 2 (IDO2) or tryptophan 2,3-dioxygenase (TDO2) in brain might occur in IDO1 KO mice. Both IDO2 and TDO2 divert tryptophan to the kynurenine pathway (Kanai et al., 2009; Qian et al., 2012). Using Real-Time quantitative polymerase chain reaction assays that detect all TDO2 and all IDO2 transcripts, their expression in brain tissue is induced following administration of LPS at a time correlated with the presence of depression-like behavior (Park et al., 2011b). Moreover, recent evidence has implicated a role of brain TDO2 in anxiety-like behavior as genetic deletion of TDO2 provided anxiolytic effects assessed as increased time spent in open areas of the elevated plus maze and open field tests (Kanai et al., 2009). For our model however, it should be noted that TDO2 activity is not impacted by the presence of 1-MT (Muller et al., 2005), therefore it is unlikely that TDO2 is compensating for decreased IDO1 activity and driving depression-like behavior observed in the current studies. To examine the possibility that compensatory actions of IDO2 may be compensating for the loss of

IDO1 we utilized the non-selective inhibitor 1-MT. Here we used a racemic mixture of both the D and L forms of 1-MT. Although it remains controversial, various models have demonstrated that levo (L)-1-MT provides significant inhibition of IDO1 and IDO2 activity (Qian et al., 2012), dextro (D)-1-MT has little effect on IDO1 activity but does inhibit IDO2 activity (Metz et al., 2007; Qian et al., 2012). Regardless of the level of inhibition of IDO1 and IDO2 provided by 1-MT, it is generally accepted that IDO1 has significantly greater enzymatic activity and likely contributes the majority of increased kynurenine in our studies (Lob et al., 2009; Qian et al., 2012).

The current data are also the first to show that 1-MT administered directly into the brain protects against depression-like behaviors and this effect is comparable to the one observed in response to systemic 1-MT (O'Connor et al., 2009c; O'Connor et al., 2009a; Salazar et al., 2012). Our findings are also in agreement with a recent publication from Dobos et al. (Dobos et al., 2012) showing that 1-MT reduced time of immobility in the forced swim test four days after a 5 μg dose of LPS was administered ICV. It is worth noting that the experimental design of the Dobos et. al. study was considerably different from the design employed here. The dose of LPS used was 500 times larger than our dose, and the timing of behavioral testing after LPS treatment was not the same. Their very high dose of LPS may cause massive cell death within the brain, and, as the authors stated, likely invoked peripheral IDO1 activity (Dobos et al., 2012). Furthermore, we injected 1-MT directly into the brain while Dobos et. al. administered 1-MT subcutaneously via a chronic release pellet or injections without determining inhibitor concentrations within the brain. Our studies were designed to more directly investigate the effects of brain IDO1 activity on neuroinflammation-dependent depression-like behavior thus all treatments were given directly into brain to minimize or eliminate peripheral effects.

Taken together our data confirm that centrally administered LPS induces depression-like behavior that occurs concurrently with elevations in brain kynurenine concentrations. Although additional experiments are necessary to fully determine the role that kynurenine metabolism plays in mediating the behavioral effects of inflammation, our data implicate IDO1 as a necessary component of central LPS-induced depression-like behavior, specifically sucrose preference. Additional, as of yet undefined, inflammatory molecules interact with IDO1 to mediate the full spectrum of depression-like behaviors as evidenced by the discrepancy observed in IDO1 KO mice submitted to TST versus the action of 1-MT on this behavior. We propose that other dioxygenases with activity similar to IDO1, such as TDO2 and possibly IDO2, play a role in increasing kynurenine levels in response to central LPS treatment. Also various depression-like behaviors may be differentially regulated by IDO1-dependent kynurenine metabolism and pro-inflammatory cytokines; warranting further investigation into these possibilities.

4.6 Figures

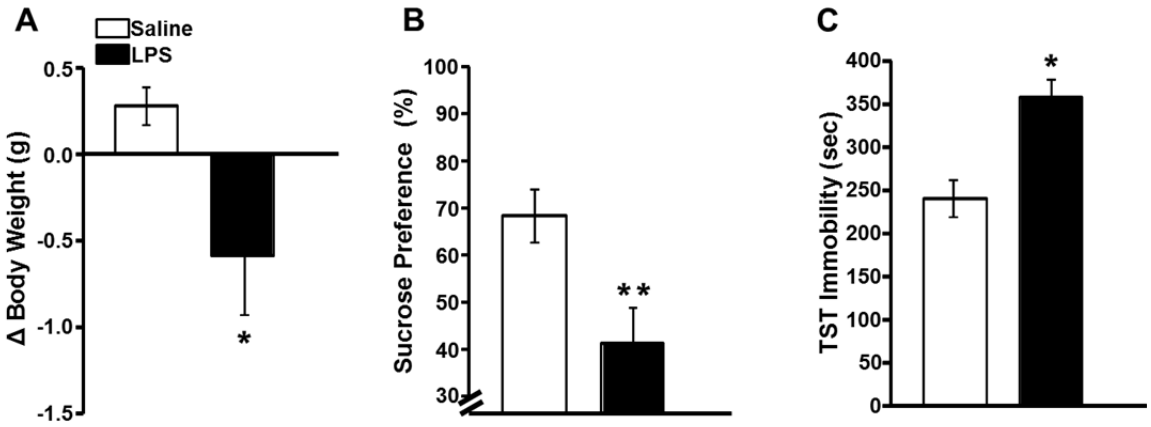

Figure 4.1: LPS administered via ICV injection induced body weight loss and depression-like behavior. A) LPS (10ng) decreased body weight over 24 h period following treatment. B) Sucrose preference was decreased by ICV LPS. C) LPS increased TST immobility. Data are average ± SEM; * $p < 0.05$, ** $p < 0.01$. n = 7 mice per group.

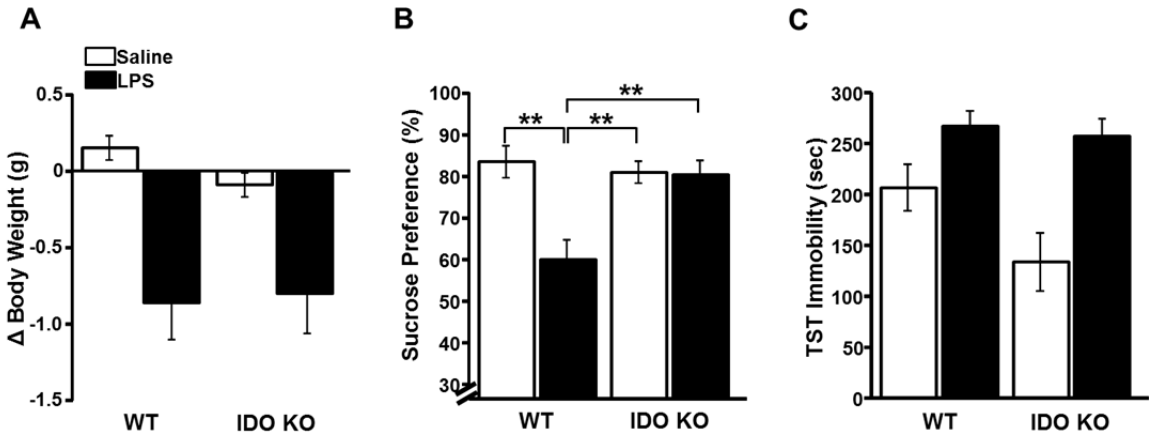

Figure 4.2: Mice deficient in IDO1 are protected from ICV LPS-induced anhedonia. A) Both WT and IDO1 KO mice had a similar decrease in body weight in response to ICV LPS. B) IDO1 KO mice maintained sucrose preference following ICV LPS treatment but had C) similarly increased TST immobility as WT mice. Data are average ± SEM; * $p < 0.05$, ** $p < 0.01$. n = 6 – 24 mice per group.

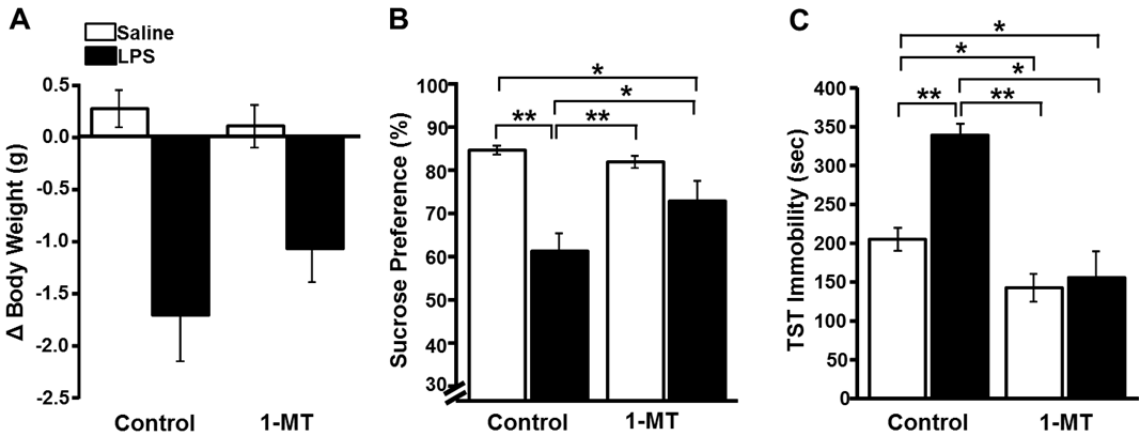

Figure 4.3: 1-MT protects mice from central LPS-induced depression-like behaviors. A) Mice given 1-MT or saline had similar decreases in body weight in response to central LPS. B) 1-MT treated mice maintained sucrose preference following ICV LPS treatment and were C) protected from LPS-induced increase in TST immobility as WT mice. Data are average ± SEM; * $p < 0.05$, ** $p < 0.01$. n = 6 mice per group.

4.7 Table

Table 4.1: Kynurenine and Tryptophan 24 h after treatment

Brain	**Control**	**LPS**
Kynurenine (nmol/mg wet wt)	0.09 ± 0.03^{a}	0.26 ± 0.04^{b}
Tryptophan (nmol/mg wet wt)	1.16 ± 0.27^{a}	1.45 ± 0.19^{a}
Kynurenine/Tryptophan	0.07 ± 0.02^{a}	0.19 ± 0.03^{b}
Plasma	**Control**	**LPS**
Kynurenine (µmol/L)	0.89 ± 0.1^{a}	0.99 ± 0.1^{a}
Tryptophan (µmol/L)	44.27 ± 1.84^{a}	47.63 ± 3.46^{a}
Kynurenine/Tryptophan	$.020 \pm 0.002^{a}$	$.021 \pm .003^{a}$

Brain and plasma were collected for analysis of kynurenine and tryptophan concentrations using HPLC. Data are average concentrations ± SEM. Averages within rows with different letters are significantly different; $p < 0.05$. n = 6-7 mice per group.

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CHAPTER 5:

Intracerebroventricular administration of HIV-1 Tat induces brain cytokine and indoleamine 2,3-dioxygenase expression: a possible mechanism for AIDS comorbid depression

5.1 Abstract

Human immunodeficiency virus (HIV) remains a major public health concern despite a large education effort during the past 25 years. A persistent problem with HIV infection is the high comorbity rate of clinical depression. We previously established that increasing proinflammatory cytokines within the brain of mice induces sickness that can culminate in depressive-like behavior. Here we investigated the role of the HIV transactivator of transcription (Tat) protein in activation of brain cytokine signaling and subsequent induction of depressive-like behavior in a murine model. Adult Balb/c mice were administered a single intracerebroventricular (ICV) injection of Tat (40 ng). Social investigation of a novel juvenile was measured at 2, 4, 8 and 24 h post-treatment. Mice treated with Tat did not display signs of sickness, as measured by either decreased social investigation or loss of body weight. At 24 h post-injection, mice were subjected to the forced swim test (FST). ICV administration of Tat to Balb/c mice increased immobility in the FST at 24 h post injection. A different strain of mice, C57BL/6J, responded similarly in the FST. Furthermore, adult C57BL/6J mice injected with Tat and tested in a two-bottle 1% sucrose preference test displayed reduced preference for sucrose during the 24 h post-injection period. Subsequently, brain tissues from Tat-treated and control C57BL/6J mice were collected at 4 and 24 h post injection. CNS tissue from Tat-treated mice had increased expression of IL-1b, TNF-a, IL-6, and IDO mRNAs at 4 h post injection.

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These data demonstrate that a single exposure to Tat in the brain is sufficient to induce brain cytokine signaling that culminates in depressive- like behavior. The results reveal a potential role for Tat in the development of comorbid depression in HIV-infected individuals.

5.2 Introduction

Human immunodeficiency virus (HIV) infection remains highly prevalent in the developed world despite significant public education efforts. The Center for Disease Control estimates that approximately 1 million people are living with HIV infections or acquired immunodeficiency syndrome (AIDS) in the United States with the rate of new infections remaining stable (CDC.gov). Comorbid depression is a significant problem among the HIV infected populationbecause these subjects have a higher incidence rate of depression than the general population (Olatunji et al., 2006). Of note, the population of individuals living with HIV infection continues to increase even with the advent of highly active antiretroviral therapy, which significantly increases the life expectancy. HIV infections with comorbid depression will likely increase as HIV treatments continue to improve.

A hallmark of HIV infection is its migration to the brain, initiating a neuroinflammatory response that contributes to the loss of neurons and AIDS dementia complex (Merrill and Chen, 1991). Immune responses in the brain during HIV infection are engaged early during infection. Infected macrophages enter the brain in low abundance during early HIV infection and activate microglial cells to initiate the production and release of cytokines (Gendelman et al., 1994). Microglia and astrocytes activated by surrounding HIV-infected cells respond with secretion of chemokines and cytokines that contribute to HIV associated dementia (Kaul et al., 2005). HIV proteins also activate macrophage-like cells in the brain, including perivascular macrophages and microglia (Rappaport et al., 1999; Kaul et al., 2001; Pu et al., 2003). The event triggers a

proinflammatory cascade that ultimately leads to increased proinflammatory cytokine expression in the brain.

Strong evidence exists for an important role of peripheral and central inflammation in the development of sickness and depressive- like behaviors (reviewed in (Anisman et al., 2002; Dantzer et al., 2008)). In addition to their activity in the brain, the proinflammatory cytokines IL-1b, TNF-a, and IFN-c activate the enzyme indoleamine 2,3-dioxygenase (IDO) (Fu et al., 2010; Henry et al., 2008; O'Connor et al., 2009b,c). IDO is a tryptophan degrading enzyme that oxidizes tryptophan to n-formylkynurenine that is then quickly converted to kynurenine. Degradation of kynurenine leads to an increase in the neuroactive metabolites quinolinic acid, 3-hydroxy kynurenine, and kynurenic acid. Evidence for increased activity of the IDO/kynurenine pathway in HIV-infected individualsis well established. The role of reduced serum tryptophan coupled with increased kynurenine in the manifestation of HIV associated neurological symptoms was presented in early research investigating peripheral macrophage activation during HIV infection (Fuchs et al., 1988). As early as 1990, Fuchs et al. provided evidence for an association between neuropsychiatric symptoms and biochemical signs of IDO activation in the plasma of HIV-infected patients (Fuchs et al., 1990). The IDO-mediated reduction in tryptophan levels observed in HIV seropositive patients was highly correlated with severity of neuropsychiatric manifestations (Werner et al., 1988). Moreover, the IDO metabolite quinolinic acid increased in the cerebrospinal fluid with HIV infection (Heyes et al., 1989) and the levels of quinolinic acid in the cerebrospinal fluid correlated well with the neurological status of patients with AIDS dementia (Heyes et al., 1991). Quinolinic acid contributes to neurotoxicity and cognitive alterations via activation of N-methyl-Daspartate (NMDA) receptors. The actions of

quinolinic acid on the NMDA receptors are antagonized by kynurenic acid,which is also increased in patients with HIV infection (Heyes et al., 1992).

Induction of IDO activity and the subsequent increase in quinolinic acid in CNS cells is not dependent on infection but instead can be caused simply via the actions of viral protein products. The HIV transactivator of transcription, Tat, is the first HIV protein produced during viral replication and is chronically expressed during HIV infection. Tat is expressed and released by cells in the brain during HIV infection, including infected astrocytes (Bruce-Keller et al., 2003; Chauhan et al., 2003). Tat is also readily taken up by brain cells using the heparan sulfate receptor in combination with the low-density lipoprotein receptor-related protein to gain entry (Li et al., 2009). Tat protein has many effects in cells of the brain, but a prominent feature is the induction of proinflammatory cytokines in both astrocytes and microglia (Mattson et al., 2005). Because Tat is able to activate IDO in vitro (Smith et al., 2001), we hypothesized that Tat would also activate IDO and induce depression-like behaviors in vivo.

5.3 Materials and methods

Animals

Sixteen male Balb/c mice aged three months purchased from Charles Rivers Laboratories (Wilmington, MA) and fifty-eight three month old C57BL/6J male mice purchased from Jackson Laboratories (Bar Harbor, ME) were allowed to acclimate to the animal care facility for at least two weeks prior to ICV cannulation surgery. Mice were individually housed and provided with ad libitum access to Teklad 8640 chow and water. Mice were housed in a temperature (23 ºC) and humidity (45%) controlled room and maintained on a 12 h light/dark cycle (lights off at 10:00 am). After the acclimation period, mice were surgically implanted with a single guide cannula (Plastics One, Roanoke, VA) directed toward the lateral ventricle. The guide cannulas

were kept clean and covered using a screw on cannula dummy for mice (Plastics One, Roanoke, VA). Coordinates for implantation were determined utilizing The Mouse Brain in Stereotaxic Coordinates (Franklin, 2001) and cannulas were placed at 1.5 mm lateral, 0.6 mm posterior, and 1.3 mm dorsal with respect to bregma. These coordinates placed the guide cannula 1 mm dorsal to the lateral ventricle. Mice were allowed to recover from surgery for two weeks before treatment. All procedures performed on the mice were in compliance with the National Institutes of Health guidelines and approved by the Institutional Animal Care and Use Committee at the University of Illinois at Urbana-Champaign.

Treatments

During the two-week recovery period, mice were handled 3–4 times per week for a few minutes to habituate them to being held and manipulated. On the day of treatment, dummy cannulas were removed and mice were injected using a single internal injector cannula for mice (Plastics One, Roanoke, VA) which extended 1 mm beyond the tip of the guide cannula to reach the lateral ventricle. Injections were administered in a volume of 1 µl using a 10 µl gas-tight syringe (SGE Incorporated, Austin, TX) over a one minute time period. Injector cannulas remained in place for at least 30 s to allow for diffusion before being withdrawn from the ventricle. Dummy cannulas were then returned to the guide cannulas immediately following the injection and prior to behavioral testing. Mice were injected at the dark cycle on the treatment day. The mice received either phosphate buffered saline (PBS) or 40 ng of recombinant HIV 1 Tat protein (kindly provided by Dr. Avindra Nath as contracted at the University of Kentucky) dissolved in PBS. This dose of Tat was selected based on data generated in hippocampal slice cultures which indicated that this dose of Tat is optimal for inducing cytokine and IDO expression (Fu et al., 2010 in preparation) and on data obtained from human astrocytes which

demonstrated that Tat (0–50 ng) induces robust IDO expression in a dose-dependent manner (Samikkannu et al., 2009). After treatment, 16 Balb/c and 40 C57BL/6J mice were submitted to behavioral testing. At the conclusion of behavioral testing, the mice were immediately sacrificed and brain tissue was collected for PCR analysis. A separate set of 18 C57BL/6J mice were injected in the same manner but were sacrificed at four h. Brain tissue was collected and prepared for real time RT-PCR analysis.

Social investigation test

To determine whether injection of Tat or vehicle induced sickness behavior, we utilized the model of social investigation. For this behavioral test, a novel unprotected juvenile mouse of approximately three weeks of age was placed in the home cage of the treated mouse. Novelty of the juvenile was maintained by ensuring that the treated mouse was never exposed to the same juvenile more than once. After placing the juvenile in the home cage, a transparent plexiglass lid was used to cover the cage to allow for an unobstructed view of the interactions. Duration of the test was five minutes, during which time interactions between the treated mouse and the juvenile mouse were video recorded for future analysis. Treated mice were tested prior to injection for a baseline measure (time 0) and then at 2, 4, 8, and 24 h post treatment. All forms of social interaction were included in the behavioral analysis, including grooming, sniffing, aggression and sexual behavior. Interactions that were initiated and maintained by the juvenile mouse exclusively were excluded. A baseline social interaction time for each mouse was established prior to the treatments and all future social interactions were compared to the baseline time (0 h, Fig. 1a).

Forced swim test

To determine whether ICV injection of Tat induces depressive-like behavior in mice, we utilized a modified version of the Porsolt forced swim test (Castagne et al., 2011). Mice were placed in a white or black plastic container (depending on mouse hair color) which was partially filled (20 cm diameter x 24 cm tall) with 24 ± 0.5 ºC water. Test duration was five min and the mice were video recorded for future analysis. The forced swim test was administered 24 h after the treatment following the conclusion of all other behavioral measures.

Sucrose preference test

To determine whether preference for sweetened solutions is impacted by exposure to Tat, we employed a two-bottle sucrose preference test. Approximately one week prior to treatment, C57BL/6J mice were trained to drink either water or 1% sucrose from two test bottles during a 24 h period. Bottles were weighed prior to being placed on the cage lids of the home cage and mice were allowed ad libitum access to the bottles. After 24 h the bottles were weighed to determine the amount of sucrose and water that had been consumed. Preference was calculated as a percentage of sucrose consumed compared to the total fluid consumption. Once all mice displayed a preference for the 1% sucrose solution, treatments were administered. The final sucrose and water bottle weights were measured immediately before the mice were submitted to the FST. On the day of treatment, 1% sucrose solution and water were provided immediately following treatment and consumption was measured over the 24 h period following injection.

Tissue collection

After mice had been subjected to the forced swim task, they were euthanized in a CO2 chamber. Mice were quickly dissected and the heart was exposed. A knick was made in the right atrium, and this was followed by rapid perfusion of ~30 ml ice-cold PBS via the left ventricle.

After the perfusion was complete brains of mice were rapidly removed and placed in a vial stored on dry ice. The brain tissue was stored frozen at -80 ºC until processing.

Tissue processing & real time RT-PCR analysis

One hemisphere of the brain tissue was removed from storage and 2 ml of cold Trizol reagent (Invitrogen, Carlsbad, CA) were added. The tissue sample in Trizol was then homogenized in the Trizol reagent using an ultrasonic tissue disruptor (Sonics and Materials Inc., Newborn, CT). The RNA was isolated according to the protocol provided with the Trizol reagent. The RNA was quantified and measured for purity (OD 260/280) using a Nanodrop instrument (Nanodrop Products, Wilmington, DE) and submitted to reverse transcription using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). These cDNA samples were analyzed using real-time quantitative RT-PCR on an Applied Biosystems Prism7900. The TaqMan gene expression assay system was used for the detection of TNF-a (catalog No. Mm00443258_m1), IL-1b (catalog No. Mm00434228_m1), IL-6 (catalog No. m00446190_m1), IDO (catalog No. Mm00492586_m1), CD11b (catalog No. Mm00434455_m1), Iba-1 (catalog No. Mm00479862_g1), MHCII (catalog No. Mm00439226_m1), GFAP (catalog No. Mm00546086_m1) and GAPDH (catalog No. Mm999999_g1). All primers were purchased from Applied Biosystems. Duplicate samples were analyzed using 125 ng of cDNA template for each reaction according to the manufacturer's instructions. Relative quantitative measurement of target gene levels was performed using the ΔΔCt method, where Ct is the threshold concentration. GAPDH was used as the endogenous housekeeping control gene.

Statistical analysis

Data are represented as the means ± SEM. All measures were analyzed using a one-way analysis of variance (ANOVA). Repeated measures ANOVA was used for social investigation, and when appropriate post hoc analysis using Fisher's protected least significant difference test was employed.

5.4 Results

Tat induces depressive-like behavior in BALB/c mice in the absence of overt sickness behavior.

To determine whether Tat protein administered ICV induces sickness behavior, we assessed the amount of time spent socially investigating a novel juvenile. Additionally, we measured body weight loss during the 24 h following treatment as a further measure of sickness in response to Tat. Tat-treated mice demonstrated no reduction in the time spent investigating a novel juvenile (repeated measures ANOVA, social investigation x treatment, Fig. 5.1A, $F_{4,52}$ = 1.9, $p > 0.05$). The 24 h changes in body weight did not differ according to treatment (Tat; 0.05 ± 0.1 g vs PBS; 0.175 ± 0.1 g; $F_{1,14} = 0.8$, $p > 0.05$). At the conclusion of the social investigation test, mice were submitted to the FST and immobility was measured as time spent floating. Mice were video recorded and behavior was scored manually by an observer blinded to the treatments. Tat-treated mice displayed increased duration of immobility at 24 h after treatment compared to control mice ($F_{1,14} = 8.4$, $p < 0.05$, Fig. 5.1B).

Tat reduces sucrose preference in C57BL/6J mice and induces depressive-like behavior.

To determine whether Tat-induced depressive-like behavior extended to mice on a different genetic background, experiments were carried out on C57BL/6J mice. These mice were submitted to the FST as well as to a sucrose preference test during the first 24 h post treatment.

Tat significantly decreased sucrose preference (Fig. 5.2A, $F_{1,26} = 5.0$, $p < 0.05$) and increased duration of immobility in the FST (Fig. 5.2B, $F_{1,26} = 7.0$, $p < 0.05$).

Tat increases proinflammatory cytokine and IDO mRNA in the brain of C57BL/6J mice.

After confirming that mice develop depressive-like behavior in response to Tat protein, we then sought to determine whether Tat caused a change in proinflammatory cytokine expression in the brain tissue of these mice at 4 h and 24 h after treatment. We employed the real-time RT-PCR to measure expression of IL- 1b, TNF-a, IL-6 and IDO mRNA in the brains of C57BL/6J mice. Tat treatment significantly increased IL-1b ($F_{1,17} = 7.8$, $p < 0.05$), TNF-a ($F_{1,17} = 5.5$, $p < 0.05$), and IL-6 mRNA ($F_{1,17} = 7.3$, $p < 0.05$) expression in the brains at 4 h but not 24 h [IL-1b ($F_{1,15} = 4.2$, $p < 0.10$); IL-6 ($F_{1,15} = 0.01$, $p > 0.05$)] post injection with the exception of TNF-a ($F_{1,15} = 4.5$, $p < 0.05$) (Fig. 5.3A). There was also a significant increase in brain IDO mRNA that was apparent at 4 h ($F_{1,16} = 9.4$, $p < 0.01$)but had subsided at 24 h ($F_{1,15} = 2.5$, $p > 0.10$) after treatment with Tat (Fig. 5.3B). Although Tat increased cytokine expression, we confirmed that a single acute dose of Tat (40 ng) protein administered ICV did not induce any neuronal damage as measured by fluorojade B labeling (data not shown).

Tat increases expression of astrocyte and microglia activation markers in the brain of C57BL/6J mice.

To assess the possibility the involvement of glia in response to Tat, we utilized real-time RT-PCR to measure expression of microglial activation markers CD11b, Iba-1, and MHC II and the astrocyte activation marker GFAP in brain tissue. Tat-treated mice displayed increased expression of CD11b ($F_{1,10} = 6.5$, $p < 0.05$), Iba-1 ($F_{1,10} = 6.2$, $p < 0.05$), MHC II ($F_{1,10} = 6.7$, $p < 0.05$), and GFAP ($F_{1,10} = 11.0$, $p < 0.01$) at 24 h after treatment (Fig. 5.3C), indicating that glial cells are activated by Tat.

5.5 Discussion

These experiments were designed to determine whether Tat given ICV can induce sickness and depressive-like behavior in mice and the possible reasons for this change. This study was based on the hypothesis that Tat, which is expressed in the brains of HIV-infected individuals (Li et al., 2009) even during HAART therapy, is able to activate brain cytokine signaling and lead to increased expressionof IDO. Our findings indicate that a single ICV injection of Tat increases brain IL-1b, TNF-a, IL-6 and IDO expression and results in depressive-like behavior but not sickness behavior.

Prior to the present study on Tat-induced depression-like behavior, the ability of Tat to induce behavioral alterations had not yet been reported, which is in contrast to other HIV protein components such as gp120. Administration of the HIV protein gp120 has been shown to induce sickness and depressive-like behaviors. For example, repeated ICV injection of the HIV protein gp120 caused a reduction in locomotor activity and a loss of body weight in rats. Further, central administration of HIV gp120 reduced preference for saccharin, which was interpreted as evidence of anhedonia (Barak et al., 2002a). These behavioral responses to central administration of gp120 were associated with increased brain expression of IL-1b and TNF-a (Barak et al., 2002b).

In the present experiment, Tat-treated mice did not display the transient sickness episode that has been observed in other models of inflammation-induced depressive-like behavior. This finding is not unique to our model of Tat-induced depressive-like behavior. Young adult mice treated centrally with a single administration of gp120 had normal social interaction behavior (Abraham et al., 2008). This was in contrast to aged mice that responded to gp120 by decreased social investigation, probably because of the increased sensitivity of their primed microglial cells

to gp120. The consideration of other indices of sickness other than loss of body weight and decreased social investigation is unlikely to account for the apparent lack of effect of Tat on sickness. These two indices have been repeatedly shown to be very sensitive to inflammatory stimuli administered either peripherally or centrally (see (Dantzer, 2001) for a review). In contrast to its inability to induce sickness behavior, Tat clearly increased duration of immobility in the FST and decreased sucrose preference, two well-accepted measures of depressive-like behavior (Porsolt et al., 1977; Willner et al., 1987).

In this model of acute Tat injection given ICV, we found that development of depressive-like behavior occurred in the absence of any major neurodegenerative change. Tat protein has been implicated as a causative agent in the neurodegenerative process associated with prolonged HIV disease (Irish et al., 2009; Li et al., 2009). Indeed, chronic exposure to Tat protein leads to profound neuronal loss as demonstrated in Tat over-expressing mice (Kim et al., 2003; Zhou et al., 2004). In order to discard this possibility, we used fluorojade B labeling 24 h after Tat injection to confirm that neurons are not undergoing degenerative events (Schmued and Hopkins, 2000).

A single acute exposure to Tat protein induced a robust increase in the expression of pro-inflammatory cytokines, as has been demonstrated by others using in vitro systems. Addition of Tat to primary human fetal astrocytes, human peripheral blood mononuclear cells, macrophages, and astrocytic and macrophage cell lines as well as rat microglial primary cultures increases the production of TNFa (Mayne et al., 1998; Nicolini et al., 2001). IL- 1b was also found to be produced by Tat-stimulated rat microglial cultures (Nicolini et al., 2001). We chose to study the expression of cytokines and IDO mRNA at the whole brain level rather than in discrete brain areas. The reasons are that we do not know yet where these molecules must be expressed in the

brain in order to induce depressive-like behavior. Secondly, brain distribution of the cellular type that is likely to be responsible for the expression of IDO, microglia, is diffuse. Many investigators study the regulation of these molecules in brain areas that are supposed to be critical for development of depression, e.g., the hippocampus, frontal cortex and basal ganglia (see for instance (Norman et al., 2010)). However, there remains a clear lack of evidence that blocking cytokine expression in these brain areas and only in these brain areas is necessary and sufficient for abrogating inflammation-induced depression. In addition, there is already evidence in the case of other brain actions of cytokines that they are expressed and act at distant sites form the brain area in which the response originates. Inflammation-induced activation of the hypothalamic–pituitary–adrenal axis is a typical example. Cytokines do not directly act on CRH-containing neurons in the paraventricular nucleus but rather act at the level of the brain stem the ascending catecholaminergic neurons originating from the ventrolateral medulla (Ericsson et al., 1994).

In the present study, Tat was able not only to increase the expression of brain proinflammatory cytokines but also to induce brain IDO expression. This effect that is consistent with what has been observed in vitro using organotypic cultures of murine hippocampal slices (Fu et al., 2010 in preparation). We measured IDO at the mRNA level because its increase is a valid marker of increased IDO enzymatic activity (Curreli et al., 2001; Andre et al., 2008). Although not tested in the context of Tat administration, we have already shown that induction of the tryptophan degrading enzyme IDO in response to inflammation is pivotal in the development of depressive-like behavior in mice (O'Connor et al., 2009a,b,c).

The lack of a sickness response to Tat occurred despite the fact that Tat induced proinflammatory cytokines in the brain. In view of this lack of effect, it is somewhat surprising

that Tat was potent enough to induce the expression of IDO and cause development of depression-like behavior. Previous experiments with lipopolysaccharide or Bacillus Calmette–Guerin have repeatedly demonstrated that increased expression of cytokines induces sickness behavior and ultimately leads to depressive-like behavior in response to activation of IDO (O'Connor et al., 2009a,b,c). It could be argued that the present dissociation between the cytokine response and sickness behavior was due to an insufficient magnitude and/or duration of the cytokine response to Tat. However, it is difficult to envision an infra-threshold cytokine response to Tat that could still lead to IDO activation and depressive-like behavior since these last two responses are supposed to be the result of a too intense or prolonged inflammatory response (Dantzer et al., 2008). An alternative interpretation is that the induction of IDO by Tat and the subsequent development of depressive-like behavior are independent of the cytokine response to Tat.

IDO is normally activated by proinflammatory cytokines including IFN-c and TNF-a. However, IFN-c independent pathways of activation of IDO have been described in response to LPS (Fujigaki et al., 2001; Wang et al., 2010) as well as to HIV infection (Maneglier et al., 2009). The IDO gene has several interferon-stimulated response elements (ISRE) and interferon-c-activated sequences (GAS) elements in its promoter region that are normally required for activation of IDO by IFN-c. The IDO promoter region also contains consensus sequences for transcriptional factors including AP-1, NF-jb and NF-IL-6 (Fujigaki et al., 2006). There is already evidence that HIV-1 Tat can activate NF-jb in brain cells both in vivo (Flora et al., 2005) and in vitro (e.g., (Nicolini et al., 2001).

The cell types in which IDO is activated in response to Tat and which could be responsible for the development of Tat-induced depression-like behavior were not investigated in

the present study. Activation of IDO and subsequent alterations in tryptophan metabolism leading to production of the excito toxin quinolinic acid in macrophages and microglia have already been proposed to play a pivotal role in neuro AIDS (Heyes et al., 1998; Smith et al., 2001). We confirm here that cells of macrophage lineage that are known to reside in the brain, including monocytes, perivascular macrophages and parenchymal microglia, display increased expression of activation markers CD11b, Iba-1 and MHC class II expression in response to Tat and are likely to be the cells responsible for producing increased IDO and cytokine expression. Astrocytes could also be implicated since they show increased GFAP expression in response to Tat and they have been shown to respond to Tat by increased IDO (Samikkannu et al., 2009).

In conclusion, these experiments establish that Tat induces a profile of activation of brain cytokines and IDO expression that leads to behavioral disturbances characteristic of human depressive symptoms. These findings confirm the possibility that a biological basis exists that contributes to the increased incidence of depression during HIV infection.

5.6 Figures

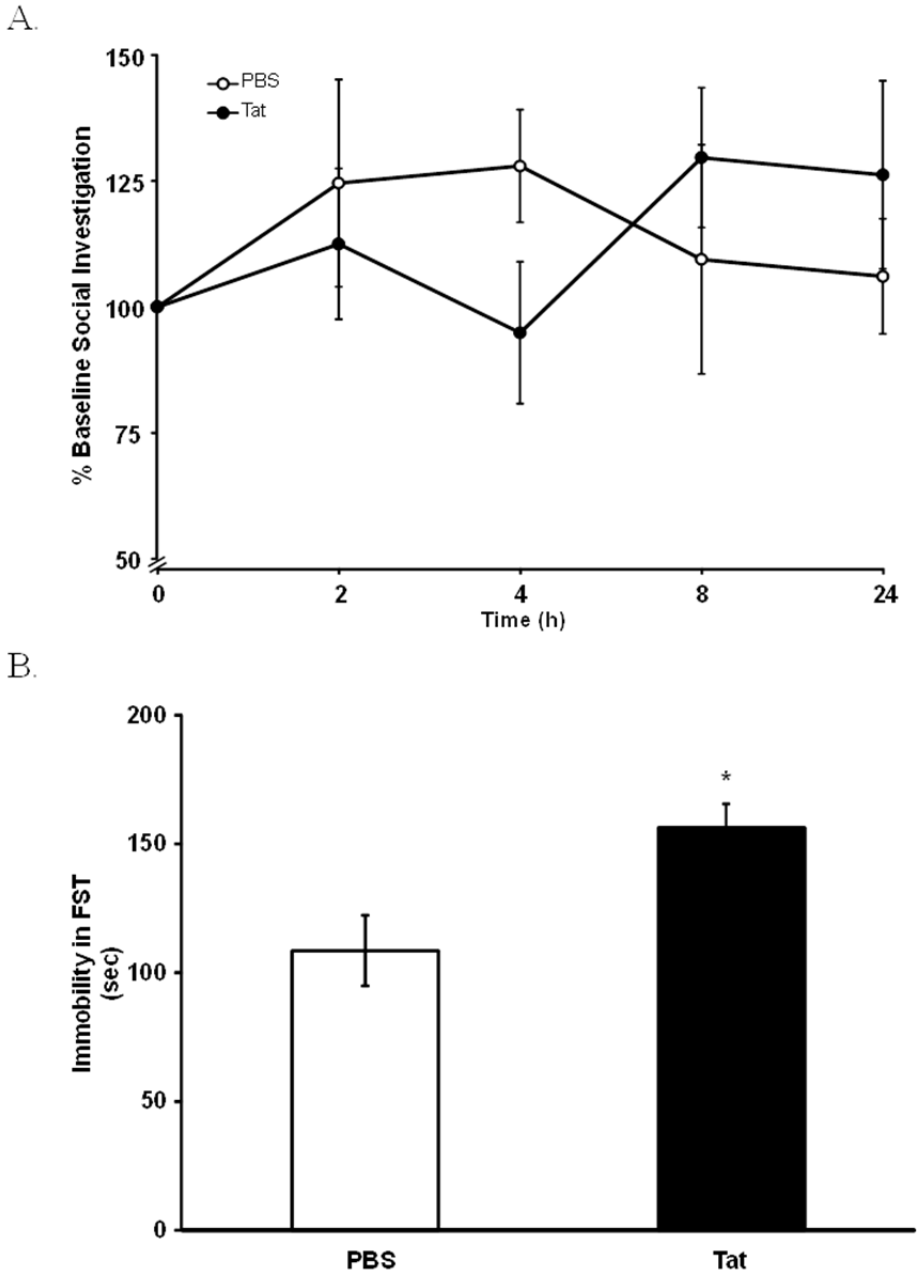

Figure 5.1: Central administration of Tat induces depressive-like behavior in the absence of overt sickness behavior. A) Tat (40 ng) protein did not cause a change ($p = 0.30$) in time spent investigating a novel juvenile measured at 2, 4, 8 and 24 h post Tat B) Tat-treated mice displayed increased ($p < 0.05$) time mice spent immobile in the FST at 24 h. Data represent mean ± S.E.M. (n = 8 mice per group).

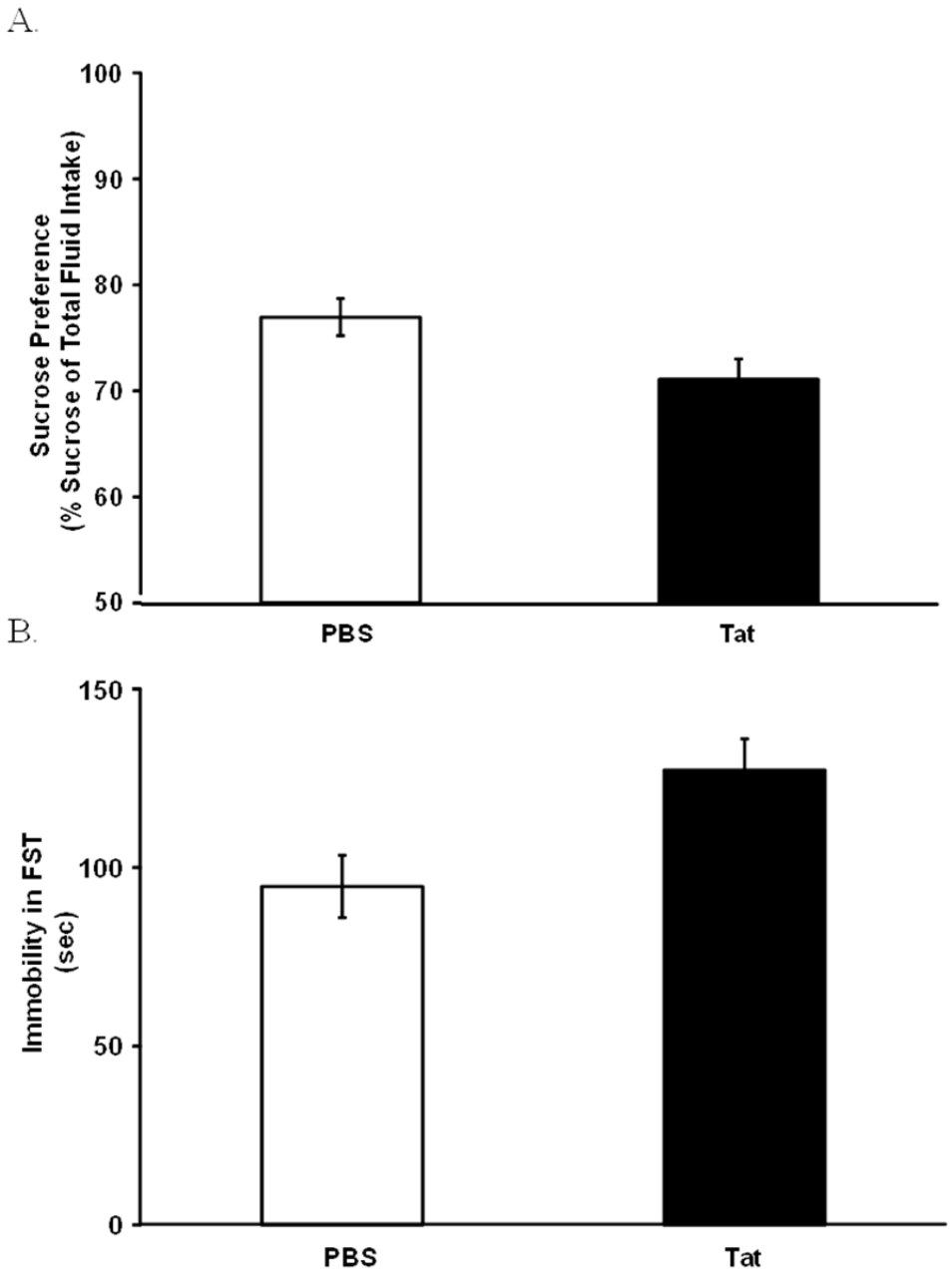

Figure 5.2: Central injection of Tat reduces sucrose preference and induces depressive-like behavior. A) Tat injection reduced ($p < 0.05$) sucrose preference compared to control injected mice. B) Tat-injected mice had increased ($p < 0.05$) immobility in the FST 24 h later. Data represent mean ± S.E.M. (n = 14 mice per group).

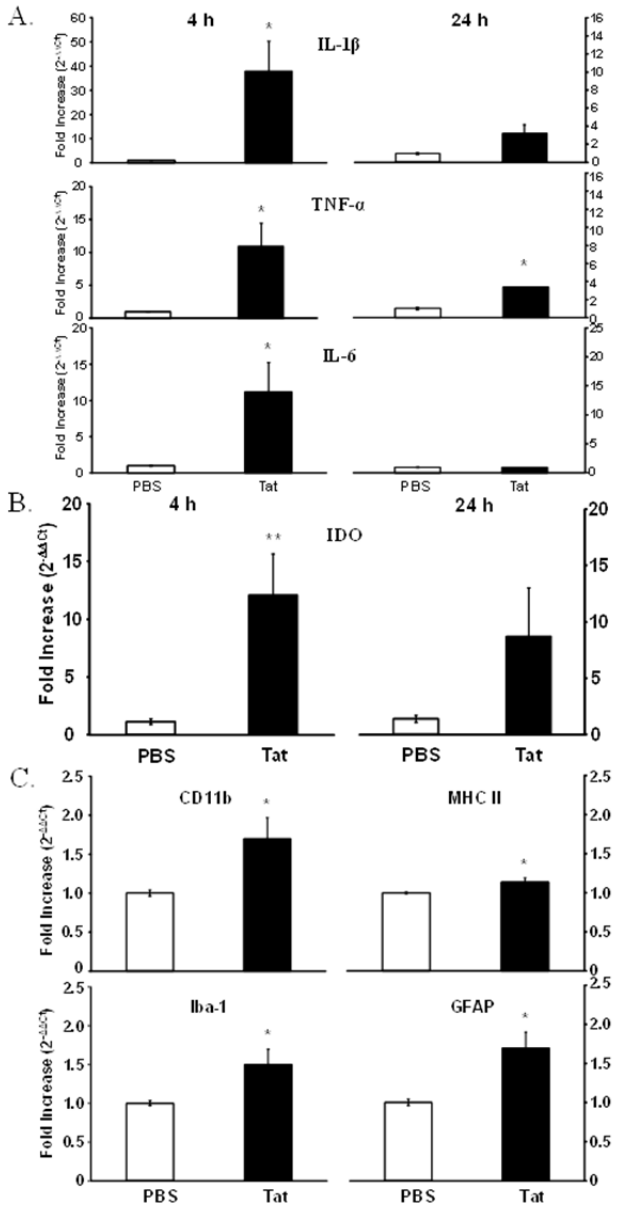

Figure 5.3: Tat protein administration into the CNS increases proinflammatory cytokine and IDO mRNA in the CNS. A) Tat injection increased brain expression of IL-1β ($p < 0.05$), TNF-α ($p < 0.05$) and IL-6 ($p < 0.05$) at 4 h post injection. Mean Ct values for PBS group were: IL-1β, 32.5 ± 0.1; TNF-α, 32.5 ± 0.1; IL-6, 32.3 ± 0.1. Tat increased TNF-α ($p = 0.05$) expression at 24 h after injection. B) IDO mRNA expression increased at 4 h after Tat treatment ($p < 0.01$; **) Data represent mean ± S.E.M. (n = 9 mice per group). Mean IDO Ct value for PBS group was 38.9 ± 0.5 C) Tat increased activation markers of microglia and astrocytes 24 h post-injection ($p < 0.05$). Data represent mean ± S.E.M. (n = 6 mice per group). Mean Ct values for PBS group were CD11b, 21.3 ± 0.3; MHCII, 22.2 ± 0.4; Iba-1, 22.3 ± 0.4; GFAP, 17.7 ± 0.4.

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Chapter 6

Summary and Future Considerations

Mood disorders and inflammation share common etiology i.e. increased expression of proinflammatory cytokines. Interestingly, depression is now strongly considered to be an inflammatory disease as the link between inflammation and mood disorders has been firmly established by the abundance of research that has been conducted (Dantzer et al., 2008; Raison and Miller, 2011; Krishnadas and Cavanagh, 2012). Neurodegenerative diseases are associated with chronic inflammation that is detectable by increased activation of glial cells accompanied by proinflammatory cytokine expression or neuroinflammation (Rothwell and Luheshi, 2000; Frank-Cannon et al., 2009). Individuals affected by neuroinflammatory diseases, including human immunodeficiency virus, have increased prevalence of comorbid depression (Ownby et al., 2010; Aznar and Knudsen, 2011; Feinstein, 2011; Aarsland et al., 2012). Thus, research directed to identify components of inflammation that represent novel therapeutic targets for major depression is becoming increasingly important. The data presented identifies two molecules that represent potential targets for treatment of neuroinflammation comorbid depression, interleukin-1 beta converting enzyme (ICE) and indoleamine 2,3-dioxygenase 1 (IDO1).

The interleukin-1 (IL-1) family of cytokines and receptors has significant influence over stress response and HPA axis (Besedovsky and del Rey, 2000), learning (Yirmiya et al., 2002) and induction of the inflammatory response (Sims and Smith, 2010). IL-1 beta (IL-1β) has received the most attention for its role in neurodegenerative disease and mood disorders (Maes et al., 1993; Allan et al., 2005; Sims and Smith, 2010; Maes et al., 2012). Direct targeting of IL-1β signaling during inflammation using IL-1 receptor antagonist (IL-1RA) has been useful for

establishing a link between brain IL-1 signaling and inflammation-induced depression (Goshen et al., 2008). However, limited transport of IL-1RA into the brain poses a challenge for treating depressed patients with intact blood brain barrier (Gutierrez et al., 1994). As such, ICE represents a novel therapeutic target for treatment of depression. To date, no research has been conducted investigating the use of ICE inhibitors for treatment of depression; however ICE activity in brain is necessary for inducing depression-like behavior in response to a neuroinflammatory challenge. Importantly, ICE activity is linked to activation of inflammasomes that respond to the presence of pathogen and danger associated molecular patterns. This makes targeting of ICE an enticing target since its activity is so intricately linked to inflammation. Pharmacologic inhibitors of ICE are already being tested to lessen damage during ischemic stroke; however it is unknown whether these inhibitors are capable of penetrating an intact blood brain barrier (Hayashi et al., 2001; Ross et al., 2007). Despite the uncertainty of their properties, preclinical testing of these inhibitors using established models of inflammation-induced depression would be informative of their potential therapeutic use since genetic deletion of ICE in humans is not logical at this time.

IDO1 has received significant research interest since its product kynurenine and also other kynurenine metabolic products have been associated with not only depression but also numerous neurodegenerative diseases that feature neuroinflammation as a disease component (Widner et al., 2000; Nemeth et al., 2006; Kwidzinski and Bechmann, 2007; Christmas et al., 2011). Although, the role of IDO1 in depression has already received tremendous research interest, questions regarding other potential roles for IDO1 remain. Of particular interest for future research is how IDO1 influences depression associated with chronic low-grade inflammation, such as that which is observed during normal aging. The aging population

represents an increasing portion of the population and as such scientists and medical care professionals will be confronted with the increasing burden of dealing with complications associated with aging. For example, many neurodegenerative diseases, such as Alzheimer's and Parkinson's disease, are associated with advance aging and as the aging population grows so does the prevalence of these diseases. As stated earlier, individuals affected by neurodegenerative disease also display a higher prevalence for comorbid depression. Neuroinflammation associated with these diseases be it causative or resulting from disease progression is associated with increased proinflammatory cytokine expression in the brain (Widner et al., 2000; Frank-Cannon et al., 2009). Limited evidence exists that examines induction of IDO1 or kynurenine metabolic products during low-grade inflammation representative of aging (Oxenkrug, 2010; Capuron et al., 2011).

The data presented indicates that both ICE and IDO1 are necessary for neuroinflammation-induced depression-like behavior. Although speculative and not investigated here, interplay between ICE/IL-1β and IDO1/kynurenine is possible despite the fact that IL-1β has only recently been identified as an inducer of IDO1 in hippocampal progenitor cells (Zunszain et al., 2012). It is worth noting that in peripheral cells IL-1 has not been shown to be a direct inducer of IDO1 (Hu et al., 1995; Fujigaki et al., 2001). Thus, going forward with this line of research, future investigation is needed to determine if and how these two enzyme systems or their products interact in vivo in brain. Once established, investigation of the interaction between these two enzyme systems will require significant research efforts to distinguish the brain regions, mechanism and outcome following the stimulation of these two systems. Despite the uncertainty surrounding this interaction the data presented establishes that ICE and IDO1 are critical players in neuroinflammation comorbid depression although significant research is still

needed to elaborate the mechanisms by which these two systems influence depression comorbid with neuroinflammation.

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